Doctoral Thesis

Medium-chain-length poly(R-3-hydroxyalkanoates): from biosynthesis towards medical applications

Author(s):
Furrer, Patrick Christian

Publication Date:
2008

Permanent Link:
https://doi.org/10.3929/ethz-a-005705895

Rights / License:
In Copyright - Non-Commercial Use Permitted
Medium-chain-length poly([R]-3-hydroxyalkanoates): from biosynthesis towards medical applications

A dissertation submitted to
ETH ZURICH
for the degree of
Doctor of Sciences

presented by
Patrick Christian Furrer
Dipl. Chem. University of Basel
born January 19, 1976
citizen of Attinghausen, UR

accepted on the recommendation of
Prof. Dr. S. Panke, examiner
Prof. Dr. T. Egli, co-examiner
Dr. M. Zinn, co-examiner

2008
For my parents
## Table of contents

**Summary**  
6  

**Zusammenfassung**  
8  

### Chapter 1: Poly([R]-3-hydroxyalkanoates): biosynthesis, recovery, and their potential for biomedical applications  
11  
  1.1 Introduction  
12  
  1.2 Biosynthesis  
13  
  1.3 Fermentative production  
18  
  1.4 Material properties  
20  
  1.5 Recovery methods  
22  
    1.5.1 Chemical digestion of non PHA-biomass  
23  
    1.5.2 Standard method: solvent extraction and precipitation of PHA in non-solvents  
26  
    1.5.3 Special approaches for the recovery of PHA  
28  
  1.6 Purification of PHA  
29  
    1.6.1 Sources and characterization of contaminations  
30  
    1.6.2 Methods to purify extracted PHA  
32  
  1.7 Potential applications of PHA in medicine and pharmacy  
34  
    1.7.1 PHA as drug carrier  
34  
    1.7.2 PHA as scaffold material in tissue engineering  
35  
  1.8 Conclusions and outlook  
37
Chapter 2: Quantitative analysis of bacterial medium-chain-length poly([R]-3-hydroxyalkanoates) by gas chromatography

2.1 Introduction  
2.2 Materials and methods  
2.3 Results and discussion  
2.4 Conclusions  
2.5 Acknowledgement  
2.6 Supplementary information

Chapter 3: Biosynthesis of medium-chain-length poly([R]-3-hydroxyalkanoate): endotoxin reduction by phosphorus limitation

3.1 Introduction  
3.2 Materials and methods  
3.3 Results and discussion  
3.4 Conclusions  
3.5 Acknowledgements

Chapter 4: Efficient recovery of low endotoxin medium-chain-length poly([R]-3-hydroxyalkanoate) from bacterial biomass

4.1 Introduction  
4.2 Materials and methods  
4.3 Results and discussion  
4.4 Conclusions  
4.5 Acknowledgement
Chapter 5: Biocompatibility of untreated and chemically modified medium-chain-length poly(3-hydroxyalkanoates) 97

5.1 Introduction 98
5.2 Materials and methods 100
5.3 Results and discussion 104
  5.3.1 Physical and chemical properties of PHAs 104
  5.3.2 Cytotoxicity according to the eluate test 105
  5.3.3 Cell culturing on PHA coatings 107
5.4 Conclusions 112

Chapter 6: General conclusions 115

References 119

Curriculum vitae 141
Summary

Poly(3-hydroxyalkanoate) (PHA) is a class of biodegradable polyesters that is accumulated by a large number of bacteria as carbon storage compound under nutrient limited growth conditions. PHA with side-chains of medium-chain-length (mcl-PHA) represents a promising class of polymers because of its broad spectrum of physical properties which are determined by the monomeric composition. Mcl-PHA has a wide variety of potential applications in medicine and pharmacy due to its versatility and biodegradability. However, a major breakthrough of mcl-PHA in the biomedical field has not taken place yet. An important reason for this is that biosynthetic products must fulfil strict requirements to be approved for medical applications. In particular biosynthesis with Gram-negative bacteria is delicate, because endotoxins from the cell membrane can contaminate the product.

In the first part of this doctoral thesis a method for the quantitative analysis of mcl-PHA was developed, to optimise the fermentative production and the downstream processing (DSP) of mcl-PHA (chapter 2). Standard methods for the quantification of short-chain-length PHA proved to be inappropriate for the analysis of mcl-PHA. It could be shown that the transesterification catalyzed by protic acids was not quantitative for mcl-PHA under typical conditions. This was due to slow reaction kinetics and the formation of side-products in case of mcl-PHA with functionalised side-chains. An improved method using boron trifluoride as catalyst was developed to quantitatively methanolyse different mcl-PHAs (recovery > 94%) and to analyse the resulting methyl esters by gas chromatography.

The fermentative production and the DSP of mcl-PHA were investigated in the second part of this doctoral thesis with the objective to generate mcl-PHA of high purity and minimal endotoxic contamination. Phosphorus limited culturing conditions were applied to Pseudomonas putida GPo1 in the chemostat to reduce the endotoxic activity of lipopolysaccharides which act as endotoxins (chapter 3). Indeed, the combination of phosphorus, carbon and nitrogen limitation drastically changed the cell composition. The content of phospholipids and the endotoxic activity of dried biomass were decreased by up to 90% under phosphorus limitation compared to conditions with excess phosphorus. Hence, the endotoxic contamination of mcl-PHA caused by the fermentation process could be drastically decreased.
To obtain mcl-PHA of high purity, the recovery from biomass was optimised (chapter 4). Classical non-solvent precipitation was replaced by applying temperature-controlled extraction and precipitation. N-hexane was found to be a suitable solvent to recover poly(3-hydroxyoctanoate) and copolymers thereof with this technique. A purity of >97% (w/w) and endotoxocities around 15 EU g\(^{-1}\) PHA were obtained. Additional re-dissolution in 2-propanol combined with temperature-controlled precipitation resulted in an enhanced purity of close to 100% (w/w) and a minimal endotoxicity of 2 EU g\(^{-1}\) PHA.

In the third part of this doctoral thesis the biocompatibility of different mcl-PHAs was studied with fibroblasts (chapter 5). Cytotoxicity experiments with aqueous eluates of mcl-PHA as well as cell-culturing experiments on mcl-PHA coatings showed that these polymers caused no toxic effect, when an effective purification procedure, such as repeated dissolution and precipitation, was applied. Cell adhesion was in fact limited on the non-polar surfaces of mcl-PHA, but chemical derivatisation of the polymer surface clearly improved its hydrophilicity and cell adhesion. However, the functionality of fibroblasts cultured on mcl-PHA surfaces seemed to be limited as a reduced collagen production was measured.

The results achieved in this work show that an optimised DSP is of immense importance to efficiently recover mcl-PHA of high purity which can be used for biomedical applications. Furthermore, the activity of endotoxins can significantly be reduced during biosynthesis of mcl-PHA which facilitates the DSP considerably. For most biomedical applications chemical modification of hydrophobic mcl-PHA is required to assure favourable interactions with cells.
Zusammenfassung


Im ersten Teil dieser Doktorarbeit wurde eine Methode zur quantitativen Analyse von mcl-PHA entwickelt, um die fermentative Produktion und die Aufarbeitung von mcl-PHA zu optimieren (Kapitel 2). Standardmethoden für die Quantifizierung von PHAs mit kurzen Seitenketten erwiesen sich als ungeeignet für die Analyse von mcl-PHAs. Es konnte gezeigt werden, dass die durch protische Säuren katalysierte Umesterung nicht quantitativ ablief für mcl-PHAs unter typischen Bedingungen. Grund dafür war die langsame Reaktionskinetik und die Bildung von Nebenprodukten im Fall von mcl-PHAs mit funktionellen Seitenketten. Eine verbesserte Methode mit Bor trifluorid als Katalysator wurde entwickelt um verschiedene mcl-PHAs quantitativ zu methanolysieren (Wiederfindung >94%) und um die resultierenden Methylester gaschromatografisch zu analysieren.

Die fermentative Produktion und die Aufarbeitung von mcl-PHA wurden im zweiten Teil dieser Doktorarbeit untersucht mit dem Ziel mcl-PHA von hoher Reinheit und minimaler endotoxischer Kontamination herzustellen. Phosphorlimitierte Wachstumsbedingungen wurden angewendet für Pseudomonas putida Gpo1 im Chemostat, um die endotoxische Aktivität der Lipopolysaccharide, welche als Endotoxine wirken, herabzusetzen (Kapitel 3). Tatsächlich wurde die Zellzusammensetzung durch kombinierte Phosphor-, Kohlenstoff- und Stickstoff-
limitation drastisch verändert. Der Phospholipidgehalt und die endotoxische Aktivität der trockenen Biomasse wurden um bis zu 90% herabgesetzt unter Phosphorlimitation verglichen mit Bedingungen mit Phosphor im Überschuss. Somit konnte die Kontamination von mcl-PHA mit Endotoxinen, verursacht durch den Fermentationsprozess, drastisch gesenkt werden.

Um mcl-PHA von hoher Reinheit zu erhalten, wurde der Aufreinigungsprozess aus der Biomasse optimiert (Kapitel 4). Die klassische Fällung des Polymers mit Hilfe eines Nicht-Lösungsmittels wurde ersetzt durch die Anwendung der temperaturkontrollierten Extraktion und Fällung. N-Hexan erwies sich als geeignetes Lösungsmittel, um Poly(3-Hydroxyoktanoat) und dessen Copolymere mit dieser Technik aufzureinigen. Eine Reinheit von >97% (w/w) und eine endotoxische Aktivität von ungefähr 15 EU g\(^{-1}\) PHA wurden damit erreicht. Zusätzliches Lösen in 2-Propanol kombiniert mit temperaturkontrollierter Fällung resultierten in einer erhöhten Reinheit von nahezu 100% (w/w) und einer minimalen endotoxischen Aktivität von 2 EU g\(^{-1}\) PHA.

Im dritten Teil dieser Doktorarbeit wurde die Biokompatibilität von verschiedenen mcl-PHAs anhand von Fibroblasten untersucht (Kapitel 5). Zytotoxizitätstests mit wässrigen Eluaten der mcl-PHAs und Zellkulturexperimente auf mcl-PHA Schichten zeigten, dass diese Polymere keinen toxischen Effekt verursachten, wenn eine effektive Aufreinigungsprozedur wie das wiederholte Lösen und Fällen angewendet wurden. Die Zelladhäsion auf unpolaren mcl-PHA Oberflächen war limitiert, doch durch chemische Derivatisierung der Polymeroberfläche konnte die Hydrophilie und die Zelladhäsion deutlich verbessert werden. Allerdings schien die Funktionalität von Fibroblasten, welche auf mcl-PHA Oberflächen kultiviert wurden, eingeschränkt zu sein, da eine reduzierte Produktion von Kollagen gemessen wurde.

Die Resultate dieser Arbeit zeigen, dass ein optimiertes Aufreinigungsverfahren von immenser Wichtigkeit ist, um mcl-PHA effizient und mit hoher Reinheit zu erhalten, welches für biomedizinische Zwecke eingesetzt werden kann. Ausserdem kann die Aktivität der Endotoxine während der Biosynthese von mcl-PHA signifikant reduziert werden, was das Aufreinigungsverfahren wesentlich vereinfacht. Für die meisten biomedizinischen Anwendungen wird eine chemische Modifikation des hydrophoben mcl-PHAs benötigt, um günstige Interaktionen mit Zellen zu gewährleisten.
Chapter 1

Poly([R]-3-hydroxyalkanoates):
Biosynthesis, recovery, and their potential
for biomedical applications

Patrick Furrer, Sven Panke and Manfred Zinn. 2007. Accepted for publication in the Handbook of Natural-based Polymers for Biomedical Applications.
1.1 Introduction

New biomaterials of the third generation are needed for particular medical applications such as vascular implants, heart valves and cardiovascular fabrics (Hench and Polak 2002; Hubbell 1995; Ueda and Tabata 2003). They are designed to stimulate specific cellular responses at the molecular level and to combine the concepts of bioactive and resorbable materials. They are supposed to help the body heal itself by prompting cells to repair their own tissues. Today’s polymeric and biodegradable systems used in medicine are mainly based on poly(lactic acid) (PLA), on poly(glycolic acid) (PGA), and on their co-polymers (Gomes and Reis 2004). Other biodegradable polymers have been proposed, but could not enter the market yet, due to lacking FDA approval (Gomes and Reis 2004).

One of the candidates is poly([R]-hydroxyalkanoate) (PHA), a class of biodegradable and biocompatible polyesters with many potential applications in the medical field, such as heart valve scaffolds (Sodian et al. 2000a; Sodian et al. 2002), pulmonary conduits (Stock et al. 2000), sutures, screws, bone plates, repair patches, stents, bone marrow scaffolds, and many others over the last years as recently reviewed by Chen and Wu (Chen and Wu 2005). PHA is composed of 3, 4, or rarely 5-hydroxy fatty acid monomers, which form linear polyesters. The general structure of poly([R]-hydroxyalkanoate) is shown in Fig. 1.1. PHAs can be separated into 3 classes according to the size of comprising monomers: short-chain-length PHAs (scl-PHA) with monomers of 3-5 carbon atoms, medium-chain-length PHAs (mcl-PHA) with monomers of 6-14 carbon atoms and long-chain-length PHAs (lcl-PHAs) with monomers of more than 14 carbon atoms. PHA is produced as reserve material by many archae and eubacteria in aerobic and anaerobic habitats. PHA accumulation occurs when microorganisms experience a metabolic stress, such as limitation by an essential nutrient in excess of a suitable carbon source (Lageveen 1986, Lee 1996b, Schlegel and Gottschalk 1962, Ward et al. 1977, Zinn and Hany 2005).

![Fig. 1.1: General structure of poly([R]-hydroxyalkanoate).](image-url)
The purity of PHA is a crucial factor for sophisticated applications. It is determined by the fermentation process and the downstream processing. The production strain and the selective recovery procedure are essential factors for obtaining PHA of high quality. Although there has been a continuous progress in downstream processing of PHA for bulk applications (more than 50 patents have been filed in the past 40 years) further improvements for high-quality PHAs and in particular for mcl-PHAs are to be expected.

The restricted availability of PHAs has been limiting research significantly. Poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are the only PHA-polymers that are currently commercially available (e.g. from Metabolix and Biomer). At present, polymers based on poly(4-hydroxybutyrate) (P4HB) are developed for medical use and one of them has been approved by FDA recently (Rizk 2005). In 2008, Telles, a joint venture between Metabolix and Archer Daniels Midland, plans to start the production of PHA under the name Mirel™ at an annual rate of 50 kt.

PHAs offer a wide range of physical properties due to their chemical diversity and due to chemical modifications of functional groups following biosynthesis.

1.2 Biosynthesis

PHAs are a class of polyesters produced as reserve materials by many archae and eubacteria (Gram-negative and Gram-positive, see Table 1.1) in aerobic and anaerobic habitats. Up to date, more than 300 microorganisms are known to synthesize PHA (Suriyamongkol et al. 2007). PHA accumulation is generally triggered when the microorganisms experience a metabolic stress, such as limitation by an essential nutrient (e.g., nitrogen, phosphorus or oxygen) and a concomitant excess of a suitable carbon source (Anderson and Dawes 1990). PHA is stored intracellularly in form of granules as a carbon and energy storage compound. The PHA granules are surrounded by a phospholipid monolayer and several proteins like the PHA polymerase and depolymerase and structural proteins called phasins are part of the granule surface (see Fig. 1.2). Upon carbon starvation or a change of the environmental pH (Ruth et al. 2007), intracellular PHA depolymerases, which are attached to the granule, release 3-hydroxyalkanoic acids.
Table 1.1: Important PHA-producing genera, substrates and resulting PHAs.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Gram stain</th>
<th>Substrates</th>
<th>PHA</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcaligenes</td>
<td>-</td>
<td>Fatty acids</td>
<td>PHB</td>
<td>Doi et al. 1992, Fukui and Doi 1998</td>
</tr>
<tr>
<td>Azospirillum</td>
<td>-</td>
<td>Fatty acids, saccharides</td>
<td>PHB</td>
<td>Itzigsohn et al. 1995</td>
</tr>
<tr>
<td>Beijerinckia</td>
<td>-</td>
<td>Glucose</td>
<td>PHB</td>
<td>Jendrossek et al. 2007</td>
</tr>
<tr>
<td>Brevundimonas</td>
<td>-</td>
<td>Saccharides</td>
<td>PHB</td>
<td>Silva et al. 2007</td>
</tr>
<tr>
<td>Burkholderia</td>
<td>-</td>
<td>Saccharides</td>
<td>PHB, PH4PE</td>
<td>Rodrigues et al. 2000</td>
</tr>
<tr>
<td>Caulobacter</td>
<td>-</td>
<td>Saccharides</td>
<td>PHB</td>
<td>Qi and Rehm 2001</td>
</tr>
<tr>
<td>Chromobacterium</td>
<td>-</td>
<td>1,3-propanediol</td>
<td>Scl- and mcl-PHA</td>
<td>Kimura et al. 2002</td>
</tr>
<tr>
<td>Clostridium</td>
<td>+</td>
<td>Acetate</td>
<td>PHB</td>
<td>Emeruwa and Hawirko 1973</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>+</td>
<td>Saccharides</td>
<td>PHBV</td>
<td>Haywood et al. 1991</td>
</tr>
<tr>
<td>Cupriavidus (former Wautersia)</td>
<td>-</td>
<td>Saccharides, fatty acids</td>
<td>PHB, PHBV, P4HB</td>
<td>Kimura et al. 1999, Vlova et al. 2006, Yan et al. 2003</td>
</tr>
<tr>
<td>Halofex</td>
<td>-</td>
<td>Saccharides</td>
<td>PHB, PHBV</td>
<td>Koller et al. 2007, Lillo and Rodriguevalera 1990</td>
</tr>
<tr>
<td>Methylobacterium</td>
<td>-</td>
<td>Alcohols, fatty acids</td>
<td>PHB, PHBV</td>
<td>Yezza et al. 2006</td>
</tr>
<tr>
<td>Nocardia</td>
<td>+</td>
<td>Fatty acids, saccharides</td>
<td>PHB, PHBV</td>
<td>Alvarez et al. 1997</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>-</td>
<td>Fatty acids, saccharides</td>
<td>PHHp - PHDd</td>
<td>Kessler and Palleroni 2000</td>
</tr>
<tr>
<td>Rhizobium</td>
<td>-</td>
<td>Saccharides</td>
<td>PHBV</td>
<td>Lakshman et al. 2004</td>
</tr>
<tr>
<td>Rhodobacter</td>
<td>-</td>
<td>Fatty acids</td>
<td>PHB, PHBV</td>
<td>Kranz et al. 1997</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>+</td>
<td>Saccharides, fatty acids</td>
<td>PHBV, PHPi</td>
<td>Füchtenbusch et al. 1998, Füchtenbusch and Steinbüchel 1999</td>
</tr>
<tr>
<td>Sphingopyxis</td>
<td>-</td>
<td>Saccharides</td>
<td>PHBV</td>
<td>Godoy et al. 2003</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>+</td>
<td>Saccharides</td>
<td>PHB</td>
<td>Szweczyk and Mikucki 1989</td>
</tr>
<tr>
<td>Streptomyces</td>
<td>+</td>
<td>Saccharides</td>
<td>PHB</td>
<td>Manna et al. 1999</td>
</tr>
</tbody>
</table>

PHB: Poly(3-hydroxybutyrate), PHBV: Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), PHPi: Poly(3-hydroxypivalate), P4HB: Poly(4-hydroxybutyrate), PHBHx: Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), P3HB-4HB: Poly(3-hydroxybutyrate-co-4-hydroxybutyrate), PHHp: Poly(3-hydroxyheptenoate), PHDd: Poly(3-hydroxydodecanoate), PH4PE: Poly(3-hydroxypent-4-enoate)
PHAs have the general chemical structure depicted in Fig.1.1. The high stereoselectivity of the producing enzyme machinery guarantees complete stereospecificity (all chiral carbon atoms in the backbone are in the \( R \) configuration), which is essential for the biodegradability and biocompatibility of PHA (Bachmann and Seebach 1999; Hocking et al. 1996). The type of bacterium and the growth conditions determine the chemical composition of PHAs and the molecular weight, which typically ranges from \( 2 \times 10^5 \) to \( 3 \times 10^6 \) Da (Byrom 1987).

Research has focused on the substrate specificity of the PHA polymerase (Rehm 2003). Four major classes of PHA polymerases have been proposed with respect to their primary structures, their substrate specificities and their subunit composition (Rehm 2007) (see Table 1.2). It was found that the substrate specificity of the PHA polymerase and the supply of cells with a particular substrate control the monomeric composition of the resulting PHA. In general, different pathways for the biosynthesis of PHA are possible (see Fig. 1.3). Acetyl-CoA activated precursors of PHA are either synthesized in the course of anabolism through \textit{de novo} fatty acid synthesis or stem in the course of catabolism from the \( \beta \)-oxidation of fatty acids that are supplied to cells.
**Introduction PHA**

**Fig. 1.3:** Metabolic routes of PHA biosynthesis. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (R)-enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-ACP reductase.

**Table 1.2:** The four classes of polyester synthases (Rehm 2003).

<table>
<thead>
<tr>
<th>Class</th>
<th>Subunits</th>
<th>Species</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>PhaC</td>
<td>Cupriavidus necator</td>
<td>$3\text{HA}<em>{\text{acL}}$-$\text{CoA}$ ($\sim$C3-C5), $4\text{HA}</em>{\text{acL}}$-$\text{CoA}$, $5\text{HA}<em>{\text{acL}}$-$\text{CoA}$, $3\text{MA}</em>{\text{acL}}$-$\text{CoA}$</td>
</tr>
<tr>
<td></td>
<td>~60-73 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>PhaC</td>
<td>Pseudomonas aeruginosa</td>
<td>$3\text{HA}_{\text{acL}}$-$\text{CoA}$ ($\sim$C5)</td>
</tr>
<tr>
<td></td>
<td>~60-65 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>PhaC, PhaF</td>
<td>Allochromatium vinosum</td>
<td>$3\text{HA}<em>{\text{acL}}$-$\text{CoA}$ ($3\text{HA}</em>{\text{acL}}$-$\text{CoA}$ [-C6-C8], $4\text{HA}<em>{\text{CoA}}$, $5\text{HA}</em>{\text{CoA}}$)</td>
</tr>
<tr>
<td></td>
<td>~40 kDa, ~40 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>PhaC, PhaR</td>
<td>Bacillus megaterium</td>
<td>$3\text{HA}_{\text{acL}}$-$\text{CoA}$</td>
</tr>
<tr>
<td></td>
<td>~40 kDa, ~22 kDa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HA-CoA: coenzyme A thioester of [R]-hydroxy fatty acids of variable length.
To date, more than 150 different hydroxyalkanoic acids are known as PHA constituents (Rehm 2007), but only few of the corresponding PHAs have been produced in large quantities and well characterized (Kessler and Witholt 1998; Witholt and Kessler 1999). As a consequence, little is known about the chemical and mechanical properties of the larger number of these polymers. To date, PHA monomers with straight, branched, saturated and unsaturated side chains have been identified (see Fig. 1.4) (Steinbüchel and Valentin 1995). Of special interest are functionalized groups in the side chain that allow further chemical modification, e.g. halogens, carboxy, hydroxy, epoxy, phenoxy, cyanophenoxy, nitrophenoxy, thiophenoxy, and methylester groups. The size of the monomer and its functional group considerably influence properties like the bioplastic’s melting point, the glass transition temperature, or the crystallinity, and therefore determine its final application.

**Fig. 1.4:** Monomers found in poly([R]-3,4 and 5-hydroxyalkanoates).
1.3 Fermentative production

Most PHA-producing bacteria start to accumulate PHA when their cell growth is impaired by the limitation of an essential nutrient (e.g., N, P, Mg, K, O or S) in the presence of excess carbon source. It is therefore important to use a suitable fermentation strategy to enhance the production of PHA. The PHA content is thereby the most important parameter for an efficient and economic downstream processing.

For fed-batch cultures a two step process is usually employed. In the first phase the cells are grown to a desired cell concentration and in the second phase PHA biosynthesis is triggered by a nutrient limitation. For continuous cultures, cell growth and PHA accumulation are controlled by the ratio of the carbon source to the limiting nutrient as well as by the dilution rate.

PHB has been efficiently produced with Alcaligenes latus in a two-step fed-batch fermentation. Nitrogen limitation was chosen as the best strategy as it allowed the greatest enhancement of PHB production. A cell concentration of 111.7 g L\(^{-1}\) and a PHB content of 88 w% were reached resulting in the productivity of 4.94 g PHB L\(^{-1}\) h\(^{-1}\) (Wang and Lee 1997a). With Cupriavidus necator (former Wautersia eutropha and Ralstonia eutropha) a PHB content of 76 w% was obtained at a cell concentration of 164 g L\(^{-1}\) resulting in a productivity of 2.42 g L\(^{-1}\) h\(^{-1}\) under nitrogen limitation (Kim et al. 1994b).

PHBV has been successfully produced with C. necator. The two-step fed-batch strategy was applied using nitrogen or phosphorus limitation in the second step. The mole fraction of HV in the co-polymer could be controlled by changing the ratio of glucose to propionic acid in the feed. PHBV contents of up to 75 w% and cell concentrations of up to 158 g L\(^{-1}\) were obtained under nitrogen limitation (Kim et al. 1994a). However, with an increasing HV mole fraction, the PHA content decreased.

P3HB4HB could be produced in fed-batch cultures under nitrogen limitation. Cell concentration of 34-49 g L\(^{-1}\) and PHA contents of 39-50 w% were reached with 4HB mole fractions of 1.6-25.2 mol% (Kim et al. 2005).

For the efficient production of scl-PHAs, recombinant E. coli and C. necator have been intensively investigated. In contrast to natural PHA-producing bacteria, PHA accumulation by recombinant E. coli was not triggered by nutrient limitation. In a fed-batch fermentation PHB was produced with recombinant E. coli reaching a cell
concentration of 204 g L\(^{-1}\) with a PHB content of 77 w\% and a productivity of 3.2 g L\(^{-1}\) h\(^{-1}\) (Wang and Lee 1997b). A higher PHB productivity of 4.63 g L\(^{-1}\) h\(^{-1}\) was obtained with recombinant \(E.\ coli\) harboring an optimally designed plasmid containing the PHA biosynthesis genes from \(A.\ latus\) (Choi et al. 1998). PHBV with 11 mol\% HV could be produced with a concentration of 159 g L\(^{-1}\) and a productivity of 2.88 g L\(^{-1}\) h\(^{-1}\) with recombinant \(E.\ coli\) (Choi and Lee 1999b). Recombinant \(C.\ necator\) was investigated for the production of PHB and PHBV but the final cell concentrations and PHA contents were only slightly increased compared to the parent strain (Lee et al. 1996; Park et al. 1995; Valentin and Steinbüchel 1995).

Although efforts have been made to use recombinant \(E.\ coli\) and recombinant \(Pseudomonas\) for the production of mcl-PHA, few fermentation processes were based on recombinant strains (Prieto et al. 1999). The production of mcl-PHA was extensively investigated with \(Pseudomonas\) species (Diniz et al. 2004; Eggink et al. 1992; Hartmann et al. 2006; Hoffmann and Rehm 2004; Hori et al. 1994; Huijberts and Eggink 1996; Kim et al. 2000). Various alkanes, alkanolic acids as well as glucose, fructose and glycerol were used as substrates for the production of mcl-PHA (Huijberts et al. 1992; Timm and Steinbüchel 1990). Especially with structurally related carbon sources, such as alkanes and aliphatic acids efficient mcl-PHA synthesis occurs (Lageveen et al. 1988). But these carbon sources are poorly water miscible or/and toxic to bacteria at rather low concentration (Sun et al. 2007). Hence the concentration of these carbon sources has to be well controlled.

For fed-batch cultures the two-step fermentation strategy has often been applied. Using octanoic acid as substrate, PHA contents up to 75 w\% at a cell concentration of 55 g L\(^{-1}\) and a productivity of 0.63 g L\(^{-1}\) h\(^{-1}\) were obtained with \(Pseudomonas\ putida\) GPo1 under nitrogen limitation (Kim 2002). With \(Pseudomonas\) IPT 046 cell concentrations of up to 50 g L\(^{-1}\) with a PHA content of 63 w\% and a productivity of 0.8 g L\(^{-1}\) h\(^{-1}\) were reached under phosphate limitation using a mixture of glucose and fructose as carbon source (Diniz et al. 2004). When oleic acid was used as substrate for the cultivation of \(Pseudomonas\ putida\) KT2442, a cell concentration of 141 g L\(^{-1}\) with a PHA content of 51 w\% and a productivity of 1.91 g L\(^{-1}\) h\(^{-1}\) were obtained under phosphate limitation (Lee et al. 2000b).

Continuous fermentation has been optimized for the efficient production of mcl-PHA (Hartmann et al. 2006; Huijberts and Eggink 1996; Prieto et al. 1999). It has
been shown that the PHA content decreased with increased specific growth rate. Thus, a compromise between PHA content and productivity is required in a single-stage continuous process (Sun et al. 2007). A two-stage continuous process was developed to overcome this limitation (Jung et al. 2001). Cell densities of 18 g L\(^{-1}\) with a PHA content of 63 w% and an overall volumetric productivity of 1.06 g L\(^{-1}\) h\(^{-1}\) were obtained.

### 1.4 Material properties

Interestingly, the material properties of PHA are similar within one class as shown in Table 1.3. They are strongly dependent on the monomeric composition, in particular on the monomers’ side-chain and on the distance between the ester linkages in the backbone. In general, PHA is water insoluble, biodegradable, and biocompatible. It can be degraded at a moderate rate (3–9 months) by many microorganisms into carbon dioxide and water using their own secreted PHA depolymerases (Jendrossek 2001). Its primary breakdown products, 3-hydroxyacids, are naturally found in animals and humans.

<table>
<thead>
<tr>
<th></th>
<th>Scl-PHA</th>
<th>Mcl-PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystallinity [%]</td>
<td>40-80</td>
<td>20-40</td>
</tr>
<tr>
<td>Glass transition temperature [°C]</td>
<td>2</td>
<td>-36</td>
</tr>
<tr>
<td>Melting point [°C]</td>
<td>80-180</td>
<td>30-80</td>
</tr>
<tr>
<td>Elongation to break [%]</td>
<td>6-10</td>
<td>300-450</td>
</tr>
<tr>
<td>Density [g cm(^{-3})]</td>
<td>1.25</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Scl-PHAs are typically crystalline thermoplasts. The homopolymer PHB is a relatively stiff and brittle bioplastic, which can be processed by melt extrusion. PHA co-polymers composed of primarily HB with a fraction of longer chain monomers, such as HV, HHx or HO, are more flexible and less brittle thermoplasts. Their structure is shown in Fig. 1.5. They can be used in melt-extrusion processes to form a wide variety of products including containers, bottles, razors, and materials for food
packaging. PHB and PHBV was used as water-proof film on the back of diaper sheets (Martini et al. 1989). PHBV is more flexible, more impact resistant and was marketed under the trade name Biopol™ by ICI/Zeneca and later on by Monsanto till 1995. Co-polymers consisting of 3-hydroxybutyrate and a 3-hydroxyalkanoic acid with at least 6 carbon atoms, for instance PHBHx was developed by Procter and Gamble and commercialized under the name Nodax. In analogy to PHBV, PHBHx is less crystalline than PHB and more flexible.

![Chemical structures of commercially important scl-PHAs: Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) and poly(4-hydroxybutyrate) (P4HB).](image)

Common mcl-PHAs are less crystalline and more elastomeric. Their properties largely depend on their side-chains. For instance, the glass transition temperature of a mcl-PHA containing linear chains up to seven carbon atoms and aromatic groups varies between -39 °C and -6 °C with an increasing content of aromatic side-chains (Hartmann et al. 2004). The physical properties of mcl-PHAs containing functional groups in the side-chains can be adapted through chemical modification. Thereby, the field of possible applications is considerably extended. Through the insertion of hydroxy groups in the side-chains of mcl-PHA, the polymer’s polarity and solubility in polar solvents could be drastically changed (Eroglu et al. 2005; Lee et al. 2000a; Renard et al. 2005). Epoxidation and crosslinking of mcl-PHAs with unsaturated side-chains increased the polymer’s tensile strength and Young’s modulus by a factor of 4 and 39, respectively (Ashby et al. 2000). Polyhedral oligomeric silsesquioxane (POSS) were linked to the side-chains of unsaturated mcl-PHAs thereby increasing the crystallinity and rising the melting point up to 120 °C (Hany et al. 2005).
Very little is known about lcl-PHA. It has been shown that the ability to polymerize long-chain-length PHAs is limited to \textit{Pseudomonas putida} KTOY06 (Liu and Chen 2007) and probably this is valid for most PHA accumulating bacteria.

1.5 Recovery methods

Recovery processes can be divided into two categories: solvent extraction and chemical digestion. In the former one PHA is extracted from biomass by using an organic solvent like methylene chloride. In the latter case the rest biomass is digested by applying chemicals like sodium hypochlorite or hydrolytic enzymes. Through cross-flow filtration or centrifugation PHA is separated then from cell debris. The main problem of chemical digestion is severe degradation of PHA, resulting in a reduction of the molecular weight.

To certify polymers for medical applications, very demanding specifications have to be fulfilled. Considerable amounts of impurities like proteins, surfactants and endotoxins are not tolerated by regulatory agencies. The presence of bacterial endotoxins in medical polymers is one of the biggest concerns of suppliers (Williams et al. 1999). Up to now only few methods for depyrogenation of PHA were described in literature, although this aspect is very important for medical applications. More details are given in section 1.6.

![Diagram of PHA recovery process](image)

\textbf{Fig. 1.6:} PHA recovery through chemical digestion (A) and solvent extraction (B).
1.5.1 Chemical digestion of non PHA-biomass

Digestion of non-PHA biomass is performed in aqueous environment without or with only small amounts of organic solvents (Fig. 1.6A). The suspended cells are usually lysed to release the PHA granules, which form an aqueous suspension. For cell lysis various techniques used in biotechnology can be applied as shown in Table 1.4. The granule envelope protects the PHA to a certain degree from chemical degradation. All the residual biomass constituents are rendered water soluble by chemical modification. This can be done by using chemical agents like peroxides, hypochlorites and ozone or by using hydrolytic enzymes. After cell disruption and digestion of residual biomass, PHA granules can be separated from cell debris by conventional methods like centrifugation or cross-flow filtration. While PHB granules have a density of about 1.2 g cm\(^{-1}\), mcl-PHA granules possess a density close to that of water (Preusting et al. 1993) and therefore do not settle in aqueous suspensions (Marchessault et al. 1995). Hence, ultracentrifugation or cross-flow filtration has to be applied (deKoning et al. 1997; Yasotha et al. 2006). Although the PHA granules are not physically dissolved in H\(_2\)O, they form a stable suspension (latex). Once the PHA granules are isolated further purification steps usually follow. In the following we will focus the discussion to the first step, the degradation of residual biomass, because this is crucial for the further processing.
**Table 1.4:** Methods to lyse bacterial cells for the recovery of PHA.

<table>
<thead>
<tr>
<th>Physical methods</th>
<th>Ref.</th>
<th>Chemical methods</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bead milling</td>
<td>Tamer et al. 1998a, Tamer et al. 1998b</td>
<td>Reducing or oxidizing agents</td>
<td>Ramsay et al. 1992, Tamer et al. 1998a</td>
</tr>
<tr>
<td>Homogenization</td>
<td>Ling et al 1997b, Tamer et al. 1998a</td>
<td>Detergents</td>
<td>de Koning et al. 1997</td>
</tr>
<tr>
<td>Ultrasonication</td>
<td>Hwang et al. 2006</td>
<td>Lytic enzymes</td>
<td>Kim et al. 1996</td>
</tr>
<tr>
<td>Supercritical fluid</td>
<td>Castor and Hong 2003, Hejazi et al. 2003</td>
<td>pH-shock</td>
<td>Tamer et al. 1998b</td>
</tr>
</tbody>
</table>

Cell debris digestion with hypochlorite (Berger et al. 1989; Hahn et al. 1994; Hahn et al. 1995; Lee and Choi 1998; Ling et al. 1997b; Middelberg et al. 1995; Tamer et al. 1998a; Taniguchi et al. 2003) was one of the earliest methods for degrading residual biomass. Most cell constituents are oxidized by hypochlorite and thus become water soluble. However, PHA was shown to become severely degraded with a decrease in molecular weight $M_w$ from e.g. 1'200 kDa to 600 kDa under optimized conditions (Berger et al. 1989; Hahn et al. 1994; Hahn et al. 1995; Ramsay 1990). In addition, it is difficult to eliminate residues of sodium hypochlorite completely in the subsequent steps. Hypochlorite digestion could be improved by combining it with surfactants or organic solvents and resulted in PHA of higher purity with less polymer degradation (Bordoloi et al. 2003; Hahn et al. 1994; Ramsay 1990).

Alternative oxidizing agents like hydrogen peroxide or ozone were investigated to oxidize residual biomass. Peroxides were especially useful to degrade nucleic acids and to decrease the viscosity of the lysate (Greer 1994; Greer 1997; Liddell and Locke 1997). In combination with enzymes and surfactants, the efficiency of rest
biomass degradation with peroxides could be increased (George and Liddell 1997). Treatment of PHA containing biomass or partially purified PHA with ozone yielded an enhanced level of purity in combination with other purification steps. The resulting polymer was basically odor-free (Horowitz and Brennan 1999). Furthermore, coloration of PHA was drastically reduced with ozone or peroxide. It has not clearly been described in literature if PHA was degraded by hydrogen peroxide or ozone. But a certain degradation has to be expected due to the strong reactivity of these agents.

Simple alkaline digestion of suspended biomass provided PHB with a purity of 85% or higher, but a minor degradation of PHA has also been observed (Choi and Lee 1999a). Combination with chelates and surfactants improved cell disruption and solubilisation of the rest biomass, and therefore PHA of higher purity was obtained.

The use of enzymes is an alternative to harsh chemical agents. Cell fragments can be degraded more selectively and the degradation of PHA is negligible. Enzyme cocktails consisting of various hydrolytic enzymes like proteinases, nucleases, phospholipases, lysozymes and others in combination with surfactants and chelate formers have been applied (Byrom 1987; George and Liddell 1997; Holmes and Lim 1990; Kim et al. 1996; Ramsay et al. 1992; Yamamoto et al. 1995). Combined with heat treatment, PHA purities of up to 95% could be achieved (deKoning and Witholt 1997). Heat accelerated the degradation of residual biomass, while surfactants and chelates improved the solubility of all components. Mcl-PHA with a purity of 93% was obtained by enzymatic digestion combined with ultrafiltration (Yasotha et al. 2006).

Generally the isolation of the PHA-granules is much easier with bacteria that have a high PHA content (>60% in dry mass). Only one or two steps are necessary to achieve a purity of around 90%. If the PHA content is below 60%, the separation process is more complicated. For this case a combined method with enzymes and reducing agents like sodium dithionite was effective (Schumann and Müller 2003). Cell components like proteins, nucleic acids and polysaccharides were decomposed without drastically changing PHA properties.

To avoid the problem of a dramatic increase in viscosity caused by the liberation of DNA, a nuclease-encoding gene was integrated into the genome of PHA
producing bacteria (Boynton et al. 1999). Thereby the amount of enzymes or chemicals necessary for the digestion of the rest biomass was reduced. The lysate viscosity was significantly reduced without affecting the PHA production or the strain stability.

Major disadvantages of the enzymatic digestion are the high costs of hydrolytic enzymes and the additional purification steps necessary to reach a high degree of purity. Moreover surfactants can hardly be separated from PHA.

The purification of PHA granules would be much more efficient if they were secreted into the medium. Separation from the cells could be done by centrifugation without digesting the residual biomass. The production of extracellular PHA has been proposed (Sabirova et al. 2006). However, this seems to be irrational as excreted PHA granules would hardly be accessible to the bacteria for a later use of PHA.

1.5.2 Standard method: solvent extraction and precipitation of PHA in non-solvents

As discussed before, the alternative method to chemical or enzymatic digestion of the residual biomass is the selective extraction of PHA from biomass with an organic solvent. Most recovery procedures based on the extraction with organic solvents follow the steps shown in Fig.1.6B. Bacterial cells are collected by centrifugation or filtration and dried to remove water that inhibits an effective extraction. Pre-treating dry biomass with methanol can be effective to remove some of the lipids and coloring impurities (Gorenflo et al. 2001; Jiang et al. 2006). PHA is extracted from the dried biomass with an organic solvent under stirring and in some cases heating. The resulting suspension is filtered or centrifuged to remove particulate cell debris and subsequently PHA is either precipitated with a non-solvent, or obtained by evaporation of the solvent. In some cases the crude PHA thus obtained is washed with a non-solvent. Repeated extraction, precipitation and washing steps result in a higher purity.

Solvent extractions normally use large amounts of solvents, typically 5-20 times of the dry weight of biomass and similar amounts of non-solvents for precipitation. Solvent recycling is energy consuming, especially for solvents with high boiling points. The high viscosity of even diluted PHA solutions (e.g. 5% w/v) impedes
extensive solvent savings and limits economical optimization. Soxhlet extraction was used to work with reduced volumes of solvent (Valappil et al. 2007).

The solubility of PHA is strongly dependent on the polymer composition, the molecular weight and on the temperature and pressure (Terada and Marchessault 1999). The extraction of scl-PHA was usually performed with chlorinated solvents like methylene chloride, chloroform, 1,2-dichloroethane, 1,1,2-trichloroethane and 1,1,2,2-tetrachloroethane (Baptist 1962b; Barham 1982; Holmes et al. 1980; Numazawa et al. 1987; Ramsay et al. 1994; Schmidt et al. 1986; Stageman 1985; Vanlautem and Gilain 1982; Vanlautem and Gilain 1987) because most chlorinated solvents are capable of dissolving PHB at a relatively high concentration, but only little of the rest biomass is dissolved as well. Preferentially the extraction was carried out at elevated temperatures to increase the solubility and to reduce the viscosity of the polymer solution and the extraction time. To reach high temperatures without evaporation of the solvent, pressurization has been applied (Kurdikar et al. 1998). PHA degradation at high extraction temperatures has been observed, especially when water was present in solution. At temperatures above 200 °C degradation occurs also in the absence of water by a non-radical random chain scission reaction (cis-elimination) (Lee et al. 2001). Recently, it has been shown that even at moderate temperatures thermal degradation occurs via E1cB mechanism if carboxylate groups are present (Kawalec et al. 2007). To precipitate the dissolved polymer, different non-solvents like methanol, ethanol, water, or ether have been used (Baptist 1962a; Holmes 1980; Kessler and Witholt 1998; Numazawa et al. 1987; Stageman 1985). Thereby, ether could only be applied for scl-PHA as it dissolves mcl-PHAs. PHB with a purity of 99% could be obtained with precipitation in water (Hrabak 1992). Mixtures of chlorinated solvents with a non-solvent were applied to extract PHB from biomass at high temperatures and to precipitate the polymer by cooling to room temperature (Schmidt et al. 1986). Azeotrope-building chlorinated solvents (e.g., 1,1,2-trichloroethane) were used for simultaneous azeotropic distillation and PHA extraction from an aqueous suspension of microorganisms (Vanlautem and Gilain 1987). Thereby water was removed from the cell suspension as a minimum boiling azeotrope. The remaining chlorinated solvent was used to extract PHA. Hence a preceding drying of the cell suspension could be avoided.
In an effort to prevent the disadvantages of halogenated solvents, which are known to pose health risks and environmental problems, alternative solvents like cyclic carbonic acid esters (Lafferty 1977), methyl lactate (Metzner et al. 1997), ethyl lactate (Metzner et al. 1997; Sela and Metzner 1996), acetic acid (Rapthel et al. 1993a; Rapthel et al. 1993b; Runkel et al. 1993a), acetic acid anhydride (Runkel et al. 1993b; Schmidt et al. 1985), n-methylpyrroloidone (Schumann and Mueller 2001), tetrahydrofuran (Matsushita et al. 1995) and mixtures of non-halogenated solvents (Noda and Schechtman 1997) were investigated for the extraction of PHB. Co-polymers with PHB were also extracted by non-chlorinated solvents like acetone (Gorenflo et al. 2001), ethyl acetate (Chen et al. 2001), butyl acetate (Walsem et al. 2004), methyl isobutylketone (Walsem et al. 2004) and cyclo-hexanone (Walsem et al. 2004).

Mcl-PHA has the advantage to be soluble in a broader spectrum of solvents than PHB. Even at room temperature typical mcl-PHAs are soluble in acetone, THF or diethyl ether. A patent from Firmenich S.A. describes an extraction method with non-halogenated solvents at room temperature tailored for polyhydroxyoctanoate (Ohleyer 1993). More recently solvents like acetone were used for extracting mcl-PHA from biomass (Jiang et al. 2006).

A combined extraction-filtration method has been described using a circular filtration system (Horowitz 2000), where the aqueous slurry was diafiltrated and an organic solvent like acetone was continuously added. In the beginning the bacterial cells were rejected, but at a certain acetone to water ratio of about 9:1 (w/w) the cells were lysed, mcl-PHA was dissolved and passed the filtration membrane.

The recycling of solvent and non-solvent mixtures is time and energy consuming. The utilisation of non-solvents can be avoided by temperature induced extraction and precipitation, described in chapter 4.

1.5.3 Special approaches for the recovery of PHA

Supercritical fluid extraction (SFE) with carbon dioxide has been proposed for the recovery of PHA (Williams et al. 1999). After extraction, carbon dioxide evaporates immediately and a drying step is not required. However, published data are conflicting. Pure PHB is soluble up to 8.01 g L$^{-1}$ in supercritical CO$_2$ at a temperature
of 348 K and a pressure of 355 bar (Khosravi-Darani et al. 2003). In contrast to these results, Seidel and Hampson showed that lipids, pigments and ubiquinones were extracted from biomass containing PHB or PHO without dissolving the polymer under similar conditions (Hampson and Ashby 1999; Seidel et al. 1991). In these reports PHA was extracted from the purified biomass with an organic solvent, e.g. chloroform (Hampson and Ashby 1999; Seidel et al. 1991). Consequently, it remains uncertain whether SFE can be efficiently used to extract PHA from biomass.

A process for recovering PHA from biological material by comminution and air classification has been patented (Noda 1995; Noda 1998). In principle no chemicals or solvent is needed for this process. The dried biomass was defatted and grinded so that the diameter of most particles was below 100 μm. An air stream was used to suspend the particles and classify them according to their weight or size. At appropriate stream velocities the particles were separated into a coarse and a fine fraction. The fine fraction was then subjected to further purification steps to reach a PHA purity of 80% or higher. So far, it has not been described whether this process is successfully applied for the recovery of PHA at a larger scale.

Furthermore dissolved-air flotation was recently used to separate mcl-PHA granules from the fermentation broth (van Hee et al. 2006). Flotation is potentially cheap and is common in wastewater treatment. Flotation separates particles according to their affinity to the air/liquid interface which is generated by bubbling air (or another gas) through a liquid phase (e.g. water). Hydrophobic particles are transported with the air-bubbles to the surface, whereas more hydrophilic ones remain in the aqueous phase. The cells were pre-treated with enzymes to release the PHA granules. Selective aggregation and flotation of mcl-PHA granules could be triggered by adjusting the pH at around 3.5. A purity of 86% was obtained after three consecutive batch flotation steps.

### 1.6 Purification of PHA

For applications in the medical field, PHA of high purity is needed. In particular biologically active contaminants like proteins and lipopolysaccharides (LPS) have to be reduced to a very low level as they could induce immunoreactions. The U.S. Food
and Drug Administration (FDA) requires the endotoxin content of medical devices not to exceed 20 U.S. Pharmacopeia (USP) endotoxin units (EU) per device, except for those devices that are in contact with the cerebrospinal fluid. In this case the content must not exceed 2.15 USP EU per device. LPS act as endotoxins whereby minute quantities can have severe effects in contact with blood and trigger immunoreactions. Particularly for PHA derived from fermentation of Gram-negative bacteria, contamination with endotoxins is a serious problem, as LPS are part of the outer membrane. During cell lysis and product recovery, LPS are liberated from the outer membrane and contaminate PHA. Therefore, PHA for use in medical devices has to be carefully purified from such endotoxins.

Standard techniques used for the purification of PHA are re-dissolution and precipitation, washing with a non-solvent, purification by chromatography, treatments with chemical agents and filtration. The purity of PHA can also be increased by washing the biomass before extracting PHA or by aqueous digestion to remove major impurities as described before.

1.6.1 Sources and characterization of contaminations

The spectrum of possible PHA-contaminants from residual biomass is broad. But basically, the extraction solvent determines which impurities will be carried over. For instance, proteins and DNA have been frequently detected when PHA was recovered by aqueous chemical digestion. Solvent extraction with non-polar solvents or with solvents of average polarity is more susceptible to co-extract lipids and coloring substances. Notably polar organic solvents like acetone and 2-propanol co-extract chromophores and give the polymer a yellow to brownish color (see Fig. 1.7). Lipopolysaccharides have been detected with aqueous digestion as well as with solvent extraction. They are soluble in water, but their amphiphilic character renders them to some extent soluble in non-polar solvents.

Besides lipids, proteins and lipopolysaccharides also antifoam agents from fermentations, surfactants and hydrolytic enzymes from the purification procedure are contaminants of the polymer. The most common impurities found in PHA are summarized in Table 1.5. In summary the nature of contaminants is determined by the biosynthesis as well as by the downstream processing.
Table 1.5: Common contaminants found in PHA.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Amount in PHA</th>
<th>Ref.</th>
<th>Recovery method</th>
<th>Microorganism PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
<td>0.1-3 mol%</td>
<td>Sevastianov et al. 2003</td>
<td>Ethanol-KOH wash + chloroform-ethanol extraction</td>
<td>R. eutropha PHB, PHBV</td>
</tr>
<tr>
<td>Proteins</td>
<td>0-9 w%</td>
<td>Ling et al. 1997a, Ling et al. 1997b</td>
<td>Homogenization + centrifugation</td>
<td>E. coli PHB</td>
</tr>
<tr>
<td></td>
<td>n.d</td>
<td>Seidel et al. 1991</td>
<td>Extraction</td>
<td>M. rhodasianum PHB</td>
</tr>
<tr>
<td>DNA</td>
<td>0.03-2.2 w%</td>
<td>Ling et al. 1997b</td>
<td>Homogenization + centrifugation</td>
<td>E. coli PHB</td>
</tr>
<tr>
<td></td>
<td>0.6-2.2 w%</td>
<td>Ling et al. 1997a</td>
<td>Homogenization + centrifugation</td>
<td>E. coli PHB</td>
</tr>
<tr>
<td>LPS</td>
<td>&gt;120 EU g⁻¹</td>
<td>Williams et al. 1999</td>
<td>N.d.</td>
<td>N.d. PHB</td>
</tr>
<tr>
<td></td>
<td>1-10⁴ EU g⁻¹</td>
<td>Lee et al. 1999</td>
<td>NaOH-digestion</td>
<td>E. coli PHB</td>
</tr>
<tr>
<td>Antifoam agents</td>
<td>&lt; 1 w%</td>
<td>Unpubl. results</td>
<td>Chloroform-ethanol extraction</td>
<td>P. putida GPo1 PHO</td>
</tr>
<tr>
<td>SDS</td>
<td>N.d</td>
<td>de Koning and Witholt 1997</td>
<td>SDS solubilisation + enzym. treatment</td>
<td>P. putida GPo1 PHO</td>
</tr>
<tr>
<td>UV absorbers</td>
<td>N.d</td>
<td>Jiang et al. 2006</td>
<td>Acetone extraction</td>
<td>P. putida mcl-KT2440 PHA</td>
</tr>
</tbody>
</table>

N.d. not defined, a) relative to the PHA monomers
Fig. 1.7: Coloring impurities co-extracted from biomass. PHO extracted with A) hexane, B) methylene chloride and C) acetone. The solvent was evaporated after filtration.

1.6.2 Methods to purify extracted PHA

Repeated dissolution and precipitation was commonly applied to reach a purity of close to 100%. The efficiency of this method is dependent on several parameters like the concentration of the polymer solution and the temperature but usually large amount of non-solvents were used. Jiang et al. (Jiang et al. 2006) showed that the concentration of UV absorbing molecules is considerably decreased by dissolution of mcl-PHA in acetone and precipitation in cold methanol as shown in Fig. 1.8. ScI-PHA was repeatedly dissolved in chloroform and precipitated in ethanol to remove biologically active substances. Traces of fatty acids from C6 to C18 were eliminated and the hemocompatibility increased (Sevastianov et al. 2003). The authors propose that these fatty acids originated from lipopolysaccharides.

Fig. 1.8: UV spectra of crude extracted and purified mcl-PHA in chloroform. 1: acetone extract precipitated in methanol, 2-4: additional cycles of dissolution and precipitation (1-3 times). The absorption bands at 275 and 241 nm were attributed to aromatic amino acids and nucleic acids (Jiang et al. 2006).
Temperature controlled dissolution and precipitation in 2-propanol was used to purify certain mcl-PHA like PHO. A purity of nearly 100 % and an endotoxicity of below 10 EU g\(^{-1}\) polymer was obtained with this method as shown in chapter 4.

To reduce the endotoxin content further to acceptable values, e.g. < 10 EU g\(^{-1}\) of PHA, a treatment with an oxidizing agent such as hydrogen peroxide or benzoyl peroxide was used successfully (Williams et al. 2001). Destruction of LPS by applying basic conditions was also successful (Lee et al. 1999; Sevastianov et al. 2003). The concentration of base and the treatment time (see Fig. 1.9) are crucial for an ideal detoxification. Otherwise, the destruction of LPS is incomplete or PHA is depolymerised and its molecular weight reduced as previously mentioned.

![Graph](image)

**Fig. 1.9:** Endotoxic activity of PHB recovered by 1.2N NaOH digestion at 30 °C for various durations (Lee et al. 1999).

Further methods can be used for purification such as washing of PHA with a non-solvent, purification by chromatography, filtration and treatment with endotoxin removing agents, however, accurate data about their efficacy are not available. For the protein purification in aqueous solutions, e.g., cationic endotoxin removal agents have been shown to be very successful (Zhang et al. 2005).
1.7 Potential applications of PHA in medicine and pharmacy

PHA has the potential to become an important compound for medical applications (Williams et al. 1999; Zinn et al. 2001). Biocompatibility and slow biodegradability are thereby essential properties. The changing PHA composition also allows favorable mechanical properties as shown in Table 1.3. In vitro cell experiments and in vivo studies have focused on PHB, PHBV, P4HB, PHBHx and PHO. An overview of the potential applications of PHA is given in Table 1.6. In the following we discuss the applications in the field of drug delivery and tissue engineering. The discussion is restricted to these fields, because the most promising research was done in these areas.

Table 1.6: Potential applications of PHA in medicine and pharmacy.

<table>
<thead>
<tr>
<th>Type of application</th>
<th>Products</th>
<th>Type of PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound management</td>
<td>Sutures, skin substitutes, nerve cuffs, surgical meshed, staples, swabs</td>
<td>Scl, mcl</td>
</tr>
<tr>
<td>Vascular system</td>
<td>Heart valves, cardiovascular fabrics, pericardial patches, vascular grafts</td>
<td>Mcl</td>
</tr>
<tr>
<td>Urology</td>
<td>Urological stents</td>
<td>Scl, mcl</td>
</tr>
<tr>
<td>Orthopaedy</td>
<td>Scaffolds for cartilage engineering, spinal cages, bone graft substitutes, meniscus regeneration, internal fixation devices (e.g. screws)</td>
<td>Scl, (mcl)</td>
</tr>
<tr>
<td>Dental</td>
<td>Barrier material for guided tissue regeneration in periodontosis</td>
<td>Scl, mcl</td>
</tr>
<tr>
<td>Computer assisted tomography and ultrasound imaging</td>
<td>Micro- and nanospheres for anticancer therapy</td>
<td>Scl, mcl</td>
</tr>
<tr>
<td>Drug delivery</td>
<td>Chemoembolizing agents, micro- and nanospheres for anticancer therapy</td>
<td>Scl, mcl</td>
</tr>
</tbody>
</table>

1.7.1 PHA as drug carrier

PHAs became candidate material as drug carriers in the early 1990s due to their inherent biocompatibility (Pouton and Akhtar 1996). Microspheres of PHB loaded with rifampicin were investigated for their use as chemoembolizing agent (agent for the selective occlusion of blood vessels) (Kassab et al. 1999; Kassab et al. 1997).
The drug release of all microspheres was very rapid with almost 90% of the drug released within 24h. The drug release rate could be controlled by the drug loading and the particle size. A similar behavior was described by Sendil and coworkers for PHBV supplemented with tetracycline (Sendil et al. 1999). PHBV was further investigated as an antibiotic-loaded carrier to treat implant-related and chronic osteomyelitis (Yagmurlu et al. 1999). The antibiotic sulbactamcefoperazone was integrated into PHBV rods and implanted into a rabbit tibia that was artificially infected by *S. aureus*. The infection subsided after 15 days and was nearly completely healed after 30 days.

In search of an efficient transdermal drug delivery system a PHO-based system with a polyamidoamine dendrimer was examined. Tamsulosin was used as the model drug. The dendrimer was found to act as weak permeability enhancer. By adding the dendrimer, the dendrimer-containing PHA matrix achieved the clinically required amount of tamsulosin permeating through the skin model (Wang et al. 2003).

### 1.7.2 PHA as scaffold material in tissue engineering

PHBV was chosen as a temporary substrate for growing retinal pigment epithelium cells as an organized monolayer before their subretinal transplantation. The surface of the PHBV film was hydrophilized by oxygen plasma treatment to increase the attachment of D407 cells to the polymer surface. The cells grew to confluency as an organized monolayer. Hence, PHBV films can be used as temporary substrates for subretinal transplantation to replace diseased or damaged retinal pigment epithelium (Tezcaner et al. 2003).

An interesting approach is the implantation of biodegradable supporting scaffolds that are seeded with tissue-engineered cells. This approach was exemplified by Sodian and co-workers (Sodian et al. 2000c) who used PHO and P4HB for the fabrication of a tri-leaflet heart valve scaffold. A porous surface was achieved with the salt leaching technique resulting in pore size between 80 and 200 µm. The scaffold was seeded with vascular cells from ovine carotid artery and subsequently tested in a pulsatile flow bioreactor. The cells formed a confluent layer on the leaflets.
In another study, the native pulmonary leaflets were resected with the use of cardiopulmonary bypass, and segments of pulmonary artery were replaced by autologous cell-seeded heart valve constructs. All animals survived the procedure without receiving any anticoagulation therapy. The tissue engineered constructs were covered with tissue and no thrombus formation was observed. It was concluded that tissue engineered heart valve scaffolds fabricated from PHO can be used for implantation in the pulmonary position with an appropriate function for 120 days in lambs (Sodian et al. 2000a).

The same group demonstrated that PHO and P4HB have thermoprocessible advantages over PGA who has better property for ovine vascular cell growth (Sodian et al. 2000b; Sodian et al. 2000c).

Vascular smooth muscle cells and endothelial cells from ovine carotid arteries were seeded on P4HB scaffolds to study autologous tissue engineered blood vessels in the descending aorta of juvenile sheep. Up to 3 months after implantation, grafts were fully patent, without any signs of dilatation, occlusion or intimal thickening. A confluent luminal endothelial cell layer was observed. In contrast, after 6 months graft displayed significant dilatation and partial thrombus formation, most likely caused by an insufficient elastic fiber synthesis (Opitz et al. 2004).

PHBHx was found to be a suitable biomaterial for osteoblast attachment, proliferation and differentiation from bone marrow cells. The cells on PHBHx scaffolds presented typical osteoblast phenotypes: round cell shape, high alkaline phosphotase (ALP) activity, strong calcium deposition, and fibrillar collagen synthesis. After incubation for 10 days, cells grown on PHBHx scaffolds were approximately 40% more than that on PHB scaffolds and 60% more than that on PLA scaffolds. ALP activity of the cells grown on PHBHx scaffolds was up to about 65 U g\(^{-1}\) scaffolds, 50% higher than that of PHB and PLA, respectively (Wang et al. 2004).

Similarly, it was observed that chondrocytes isolated from rabbit articular cartilage proliferated better on PHB scaffolds blended with PHBHx than on pure PHB scaffolds. Chondrocytes proliferated on the PHB-PHBHx scaffold and preserved their phenotype up to 28 days (Deng et al. 2003).
1.8 Conclusions and Outlook

Polyhydroxyalkanoates with a wide range of physical properties are accessible through biosynthesis in bacteria. It has been shown that they have a potential in several medical applications. Unfortunately, inappropriate downstream processing of the polymer resulted in contamination of PHAs by bacterial cell compounds and therefore may have affected first studies in a negative way. Improved purification methods have been described in the last years which were successful in reducing pyrogenic contaminations. Recently, a type of PHA, P4HB, obtained the approval of the U.S. Federal Drug Administration for application as a suture material. It is to be expected that more PHAs will follow because material properties of PHAs can already be tailored for particular applications during biosynthesis or later on by chemical and physical modifications.
Chapter 2

Quantitative analysis of bacterial medium-chain-length poly(R-3-hydroxyalkanoates) by gas chromatography

2.1 Introduction

Polyhydroxyalkanoates (PHAs) are a class of biodegradable and biocompatible polyesters with many potential applications in the medical field (Chen et al. 2005). In particular, the scientific and industrial research on elastomeric PHA with medium-chain-length side chains (mcl-PHA, containing 3-hydroxyalkanoate monomers with chain lengths from C₆-C₁₄) has been intensified in the last years (Chen et al. 2005; Sodian et al. 2000; Stock 2000; Williams et al. 1999; Zinn et al. 2001).

PHAs are produced by microorganisms under unbalanced growth conditions (Anderson and Dawes 1990). In order to control and optimize the conditions of fermentation and down-stream processing, the quantitative analysis of PHA in biomass and of purified polymer is of great importance. Different methods have been applied to determine the content of PHA in biomass and to analyze the monomeric composition of PHA-copolymers. After the discovery of poly(3-hydroxybutyrate) (PHB), a short-chain-length PHA (scl-PHA), Lemoigne saponified the extracted PHB, and determined the amount of PHB by gravimetry after applying a complicated treatment (Lemoigne 1926). Slepecky and Law converted PHB with concentrated sulphuric acid to crotonic acid and estimated the content via its UV absorption at 235 nm (Slepecky and Law 1960). Recently, Fourier transform infrared spectroscopy (FTIR) has been applied to determine the content of PHA in cell suspensions (Jarute et al. 2004; Randriamahefa et al. 2003). However, all these methods lack the specificity to discriminate between different monomers and hence they can not be used to determine the monomeric composition of PHA copolymers.

¹H and ¹³C NMR experiments were applied to scl- and mcl-PHA without chemical derivatisation of the polymer (Doi et al. 1995; Doi et al. 1986; Jan et al. 1996). For quantitative measurements, NMR is limited to purified PHA. For scl-PHA, ¹H NMR measurements are sufficient to elucidate their composition, whereas for mcl-PHA time-consuming ¹³C-NMR measurements are necessary. The need for faster analytical methods for the determination of PHA in biomass directly forced the development of chromatographic methods such as high performance liquid chromatography (HPLC) or gas chromatography (GC). This requires a quantitative depolymerisation of the polymer, usually combined with a derivatisation. Ion-exchange HPLC with conductivity detection was applied for the analysis of digested
poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) in activated sludge (Hesselmann et al. 1999). However, the use of GC is more common due to the simple detection by flame ionisation (FID). The first derivatisation methods for GC were developed for PHB (Braunegg et al. 1978; Riis and Mai 1988). Sulphuric acid in methanol and later hydrochloric acid in n-propanol were used as reagent for the transesterification, forming methyl and propyl esters, respectively. In contrast to the alkaline hydrolysis (Wallen and Rohwedder 1974), which led to a mixture of 3-hydroxyalkanoic acid methyl esters and 2-alkenoic acid methyl esters, the acidic transesterification resulted in the ideal case in only one methyl ester per component. In addition, it was considerably faster because extraction and transesterification proceeded in one step. Certain modifications have been introduced for the acidic transesterification method of PHA in biomass, like an extended reaction time for the analysis of PHB (Huijberts et al. 1994; Jan et al. 1995), and an increased concentration of sulphuric acid for scl-PHA (Oehmen et al. 2005) and mcl-PHA (Lageveen et al. 1988; Lee and Choi 1995). For mcl-PHA, sulphuric acid in methanol was used (Huijberts et al. 1994; Lageveen et al. 1988; Lee and Choi 1995). However, sulphuric acid is of limited use as a general catalyst for transesterification reactions, due to further decomposition of the 3-hydroxy esters (Oehmen et al. 2005; Riis and Mai 1988), e.g. by acid catalyzed elimination.

So far, the transesterification of mcl-PHA has not been studied in detail. In this work, we show that the methods developed for scl-PHA can not be adapted to mcl-PHA without reservation. This is because the reaction kinetics differ significantly between scl- and mcl-PHAs and side products occur for certain side-chain functionalized mcl-PHAs, e.g. olefinic mcl-PHAs. Both effects lead to a significant underestimation of the effective PHA content and to an incorrect copolymer composition. The aim of this work was to develop a novel method for the accurate quantification of mcl-PHA by GC for purified polymer as well as for mcl-PHA in biomass directly. We found that the Lewis acid boron trifluoride ($\text{BF}_3$) in methanol is an effective transesterification reagent without significant formation of side-products. We attribute this mainly due to the avoidance of water and protic acids. $\text{BF}_3$ in methanol is well known for the derivatisation of fatty acids (Lopez-Lopez et al. 2001; Moffat et al. 1991; Rotzsche 1991; Shantha et al. 1993). The transesterification with $\text{BF}_3$-methanol was quantitative under optimized conditions for all mcl-PHAs analyzed.
in this study, and the copolymer composition could be confirmed by NMR measurements.

2.2 Materials and methods

PHA derivatisation. The recovery and the composition of different purified PHAs and of PHAs in biomass directly was determined by GC after their depolymerisation and derivatisation to methyl or propyl esters. Each sample was derivatised twice for independent analysis. The propanolysis according to Riis and Mai was applied to a set of scl- and mcl-PHA as described by Riis and Mai (Riis and Mai 1988).

Further, this method was adapted for mcl-PHA to improve the recovery of propyl esters. The following conditions were used: About 10 mg of polymer was transferred into a 10 mL Pyrex tube. One mL of methylene chloride containing 10 mg mL$^{-1}$ of 2-ethyl-2-hydroxybutyric acid as internal standard was added and the polymer was let to dissolve at room temperature for one hour. One mL of a 20/80 (v/v) mixture of HCl (37%) and n-propanol was added, the tube was tightly sealed, and the mixture was vigorously shaken. The tube was placed in an oven at 80 °C for 16 h. Subsequently, it was cooled to room temperature and 2 mL of demineralized water was added. The mixture was vigorously shaken and after phase separation, the aqueous (upper) phase was removed. The organic phase was dried over Na$_2$SO$_4$, neutralized by adding Na$_2$CO$_3$, and filtered through a 1.0 µm nylon filter. In contrast to the original method (Riis and Mai 1988), we chose a lower reaction temperature but an extended reaction time. Furthermore, methylene chloride was used as solvent instead of 1,2-dichloroethane and 2-ethyl-2-hydroxybutyric acid was used as internal standard instead of benzoic acid. Additionally, the organic phase was dried and neutralized to remove residual water and acid.

A similar transesterification procedure was developed using boron trifluoride (BF$_3$) as catalyst. In contrast to the transesterification catalyzed by hydrochloric acid, the transesterification catalyzed by BF$_3$ did not work with n-propanol. Therefore methanol was used instead, forming methyl esters. About 10 mg of polymer was transferred into a 10 mL Pyrex tube. One mL of methylene chloride containing 10 mg mL$^{-1}$ of 2-ethyl-2-hydroxybutyric acid as internal standard was added. The polymer was let to dissolve at room temperature for one hour. One mL of BF$_3$ (1.3 M)
in methanol was added, the tube was tightly sealed and the mixture was vigorously shaken. The tube was placed in an oven and kept at 80 °C for 20 h. The subsequent extraction and drying procedure was similar to the procedure described above, except that the sample was extracted twice with 1 mL of a saturated NaCl solution for a complete removal of the catalyst. Method optimization was carried out with 50 mg of polymer and corresponding volumes of solvent and reagent to decrease the measurement uncertainty. The measurement uncertainty was calculated from the standard deviation of the transesterification (n=8) combined with uncertainties inherent to the analytical system. Thereby, the mass of derivatised PHA was important. The transesterification of larger masses (50 mg instead of 10 mg) decreased the measurement uncertainty from ± 7.2% to ± 4.0%.

For the direct analysis of PHA, 50 mg biomass was derivatised in the same way as described for 10 mg of purified PHA. For comparison, 5 g of biomass was extracted twice with 100 mL methylene chloride for 24 h. The PHA solution was filtrated, concentrated to 20% (v/v) and the polymer was precipitated by adding 200 mL of ethanol at 0 °C. The polymer was dried under vacuum at 40 °C for two days.

**Analytical methods.** For gas chromatographic analysis, one µL of the ester solution was analyzed on a GC (GC 8575 Mega 2, Fisons Instruments, Rodano, Italy) equipped with a Supelco SPB-35 (30 m x 0.32 mm) column with a film thickness of 0.25 µm (Supelco, Bellefonte, USA) at a split ratio of 1:10 and an initial temperature of 80 °C. The temperature was raised with a rate of 10 °C min⁻¹ to 240 °C. For quantification purposes by FID, known amounts of pure 3-hydroxybutyric, -valeric, -hexanoic, -octanoic, -nonanoic and -undecanoic acid were derivatised and measured to calculate their response factors. 3-Hydroxyalkanoic acids were not available commercially and their response factors were assumed to be similar to those of their corresponding saturated alkanoic acids. The response factor of 3-hydroxy-6-heptenoic acid was calculated by linear interpolation. 2-Ethyl-2-hydroxybutyric acid (Sigma-Aldrich, Buchs, Switzerland) was used as internal standard because of its chemical similarity to the 3-hydroxyalkanoic acids and because its elution time is different to the 3-hydroxyalkanoic acids measured. Tetradecane (Sigma-Aldrich, Buchs, Switzerland) was used as second internal standard. 3-Hydroxyalkanoic acids were purchased from Larodan (Malmö, Sweden).
NMR experiments in solution were performed on a Bruker AV-400 spectrometer at 297 K using a 5 mm broad-band probe. For PHBV samples, typically 5 mg polymer were dissolved in 0.7 mL CDCl₃, for purified mcl-PHA samples, 30 mg per 0.7 mL CDCl₃ were used. NMR samples of whole cells were prepared by stirring 50 mg biomass in 1 mL CDCl₃ for 5 h followed by filtration. Chemical shifts are given in ppm relative to the remaining signals of chloroform as internal reference ($^1$H NMR: 7.26 ppm; $^{13}$C NMR: 77.0 ppm). Proton ($^1$H) NMR spectra were recorded at 400.13 MHz with a 9.6 $\mu$s 90° pulse length, 4460 Hz spectral width, 64 k data points, and 24 scans with a relaxation delay of 20 s were accumulated. Carbon ($^{13}$C) NMR spectra at 100.61 MHz with $^1$H WALTZ decoupling were recorded for the mcl-PHA samples with the following parameters: 3.2 $\mu$s 45° pulse length, 26250 Hz spectral width, 64 k data points, 5000 scans, relaxation delay 10 s, and decoupling field 2.5 kHz. $^{13}$C NMR chemical shifts of PHA monomer units are compiled in the Supplementary Information.

Propyl and methyl esters and side-products were analyzed by HPLC on a C18 Nucleosil column (2 x 250 mm, 3 $\mu$m, 100 Å, Macherey-Nagel Inc., Easton, USA). Separation was achieved using a linear gradient from 10% to 80% acetonitrile in 20 min. 0.1% acetic acid in double distilled water was used as mobile phase. The flow rate was 0.2 mL min$^{-1}$ with injection volumes of 7.5 $\mu$L. Sample compounds were identified by mass spectroscopy with an APCI ion source and an ion trap mass detector (esquire HCT, Bruker Daltonics, Bremen, Germany).

To describe the monomeric composition of PHAs, the notation Cn:x was used, where n is the number of carbon atoms and x indicates the position of the double bond.

**PHA biosynthesis.** The following PHAs were produced by cultivation of *Cupriavidus necator* for scl-PHA and with *Pseudomonas putida* GP01 for mcl-PHA as described elsewhere (Hartmann et al. 2006; Zinn et al. 2003) (see Table 2.1): Poly(3-hydroxybutyrate) (PHB), from a carbon feed of 100% butyric acid; poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), from a carbon feed of 80% (mole mole$^{-1}$) butyric acid and 20% (mole mole$^{-1}$) valeric acid (PHBV-a), and 100% valeric acid (PHBV-b); poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) (PHO), from a carbon feed of 100% octanoic acid; poly(3-hydroxy-10-undecenoate-co-3-hydroxy-8-
nononoate-co-3-hydroxyoctanoate-co-3-hydroxy-6-heptenoate-co-3-hydroxy-hexanoate) (PHOUE), from a carbon feed of 46% (mole mole\(^{-1}\)) octanoic acid and 54% (mole mole\(^{-1}\)) 10-undecenoic acid; poly(3-hydroxy-10-undecenoate-co-3-hydroxy-8-nononoate-co-3-hydroxy-6-heptenoate) (PHUE), from a carbon feed of 100% 10-undecenoic acid; and poly(3-hydroxyundecanoate-co-3-hydroxynonanoate-co-3-hydroxyheptanoate-co-3-hydroxypentanoate) (PHUA), from a carbon feed of 100% undecanoic acid. Polymers were purified by repeated extraction and precipitation with methylene chloride and ethanol, respectively.

2.3 Results and discussion

The propanolysis method developed by Riis and Mai (Riis and Mai 1988) was applied to various scl- and mcl-PHA. The data obtained with GC-FID were compared with the results from NMR as shown in Table 2.1.

The monomeric composition of PHBV copolymers was determined from quantitative \(^1\)H NMR spectra. The signals of the methyl groups of HB units at 1.28 ppm and of HV units at 0.90 ppm are well separated, and the ratio between these intensities was used to calculate the polymer composition. The overall intensities of the methine HB- and HV- resonances at 5.26 and 5.16 ppm such as that of the backbone methylene protons resonating at 2.54 ppm were used to cross-check the individual HB and HV fractions.

The copolymer composition of mcl-PHAs was also investigated with NMR spectroscopy. The combined fractions of (C6:0 + C8:0) units and (C7:6 + C9:8 + C11:10) units were readily obtained from \(^1\)H NMR spectra by integration of the overlapping methyl group signals at 0.87 ppm and the olefinic proton signals at 5.78 ppm. For standardization, the intensities of the backbone –CH protons resonating at 5.17 ppm was set to 100 (mol%). The signals of the different saturated and unsaturated PHA units overlap in the \(^1\)H NMR spectrum, but the resonances are separated in the corresponding \(^{13}\)C NMR spectrum (Supplementary Information Table 2.3). In these spectra, the intensity of the backbone -CH carbons resonating at 69.8 – 71.3 was then set to 100 (mol%). This resulted in relative intensities of 99 - 103 for the overlapping carbonyl carbons at 168.8 – 169.8 ppm and of 96 - 101 for the backbone methylene carbons at 39.1 ppm. From this good agreement between
the intensities for backbone carbons C1, C2 and C3 we concluded that a relaxation delay of 10 s between individual pulses was sufficient to yield quantitative $^{13}$C NMR data, and the intensities of resolved carbon signals of different monomer units were used to determine the individual fractions. For example for sample PHOUE, integration of the resonances at 13.9, 22.5 and 31.5 ppm for C8:0 yielded an intensity of $(32.9 \pm 1.7)$. Similarly, fractions of C6:0 = $(7.1 \pm 0.8)$, C7:6 = $(12.7 \pm 0.2)$, C9:8 = $(38.2 \pm 0.3)$ and C11:10 = $(9.1 \pm 0.4)$ were determined. The sum of C6:0 and C8:0 units is 40 mol%, close to the 41.3 mol% determined from the $^1$H NMR spectrum. Also for the sum of unsaturated monomer units, the agreement between the $^{13}$C NMR (60 mol%) and $^1$H NMR (58.7 mol%) data is satisfactory. NMR results were converted to % w/w for direct comparison with GC data.

The original propanolysis method was quantitative for the transesterification of PHB and PHBV containing a low amount of valerate. Nearly 100% of the polymer was recovered as propyl ester. However, for PHBV with a high amount of valerate and for PHAs with longer side-chains the reaction was not quantitative and the recovery of propyl esters fell below 50% for PHU. The reaction time was not sufficient for a complete reaction of mcl-PHA and side-products were observed for PHA with unsaturated side-chains.

Therefore we tried to improve the HCl-propanol method for PHO and PHUE, two common mcl-PHAs (Bear et al. 1999; Dufresne et al. 2001). Experiments with these two co-polymers revealed that the maximum recovery of 97% for PHO, and 55% for PHUE was reached after 16 h of propanolysis at 80 °C. Increasing the reaction temperature or reaction time decreased the recovery of PHUE due to the formation of side-products. Already after 16 h side-products were observed for PHUE in the GC-chromatogram as shown in Fig. 2.1a. In contrast to PHUE the recovery of PHO reached a plateau after 16 h and no considerable side-products were observed. The improved HCl-propanol transesterification method (16 h at 80 °C, [HCl] = 1.2 M) was quantitative for scl- and mcl-PHAs with saturated side-chains. Table 2.1 shows that the recovery had significantly decreased for PHA containing unsaturated side-chains, due to the formation of side-products (see Fig. 2.1a). Thus HCl-propanol is an effective reagent for the transesterification of all PHAs with saturated side-chains under these conditions.
Table 2.1: Monomeric composition and recovery (% w/w) of different scl- and mcl-PHA analyzed with different GC-transesterification methods and with $^1$H NMR for scl–PHA and $^{13}$C-NMR for mcl-PHA, respectively. For GC-measurements with 10 mg of PHA a measurement uncertainty of ± 7.2% was determined.

<table>
<thead>
<tr>
<th>PHA$^a$</th>
<th>Analysis</th>
<th>C4:0</th>
<th>C5:0</th>
<th>C6:0</th>
<th>C7:0</th>
<th>C8:0</th>
<th>C9:0</th>
<th>C11:0</th>
<th>C7:6</th>
<th>C9:8</th>
<th>C11:10</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td>NMR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BF$_3$-MeOH</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^1$</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^2$</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PHBV-a</td>
<td>NMR</td>
<td>81</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BF$_3$-MeOH</td>
<td>34</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^1$</td>
<td>79</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^2$</td>
<td>78</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>97</td>
</tr>
<tr>
<td>PHBV-b</td>
<td>NMR</td>
<td>35</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BF$_3$-MeOH</td>
<td>17</td>
<td>48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^1$</td>
<td>33</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^2$</td>
<td>29</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>PHO</td>
<td>NMR</td>
<td>14</td>
<td>86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BF$_3$-MeOH</td>
<td>11</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^1$</td>
<td>12</td>
<td>85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^2$</td>
<td>9</td>
<td>64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>PHOUE</td>
<td>NMR</td>
<td>6</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BF$_3$-MeOH</td>
<td>4</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^1$</td>
<td>5</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^2$</td>
<td>4</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>PHUE</td>
<td>NMR</td>
<td>23</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>BF$_3$-MeOH</td>
<td>22</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^1$</td>
<td>6</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^2$</td>
<td>8</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>PHUA</td>
<td>NMR</td>
<td>1</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BF$_3$-MeOH</td>
<td>2</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^1$</td>
<td>2</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>HCl-PrOH$^2$</td>
<td>2</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>66</td>
</tr>
</tbody>
</table>

$^a$Fermentation conditions see materials and methods.

$^1$Transesterification method with HCl-propanol adapted for mcl-PHA: 16h, 80 °C, [HCl]=1.2 M.

$^2$Transesterification method with HCl-propanol developed by Riis and Mai for PHB.
Fig. 2.1: GC-FID-chromatograms of PHUE transesterified with the adapted HCl-propanol method of this work (a) and with BF$_3$-methanol (b).

We suspected the C7:6-monomer to excessively undergo side-reactions during propanolysis and to form the side-products observed by GC. To confirm this hypothesis and to elucidate the structure of the side-products, 3-hydroxy-6-heptenoic acid produced by fermentation and separated by column chromatography (Ren et al. 2005) was propanolysed for 2 days with HCl-propanol at 100 °C to quantitatively convert it into side-products. LC-MS measurements showed that these products had identical retention times and mass spectra as the dominant side-products from PHUE obtained by transesterification with HCl-propanol. Two products with a molecular mass of 186 u were detected with LC-MS, both possessing the same fragmentation pattern. NMR experiments revealed that the two products were (2- S, 5- R) and (2- R, 5- R) diastereomers of (5-methyl-tetrahydrofuran-2-yl)-acetic acid propyl ester as shown in the supplementary information, Fig. 2.8.

These side-products were generated by the intramolecular cyclisation of propyl 3-hydroxy-6-heptenoate. We propose that a secondary cation is formed, catalysed by protic acids and that the furane ring is built by an intramolecular nucleophilic attack, as shown in Fig. 2.2A. The chiral centre in position 5 is in R configuration due to biosynthesis whereas the chiral centre in position 2 is generated during cyclisation. The ratio between the (2- S, 5- R) and (2- R, 5- R) diastereomers is 69:31 according to $^1$H NMR. The chirality in position 5 induces asymmetry in the new chiral centre in position 2. The formation of pyrane analogs was not observed, since the formation of an intermediate primary cation is less favoured. Likewise, the formation of 7- and 9-
membered rings from propyl 3-hydroxy-8-nonenolate and propyl 3-hydroxy-10-undecenolate was not observed. The addition of \( \text{H}_2\text{O} \) to the double bond led to further side-products. This reaction most likely proceeds again through the secondary cation. Subsequently, \( \text{H}_2\text{O} \) acts as nucleophile to generate a secondary alcohol as shown in Fig. 2.2B. According to the NMR data for PHU, 32% and 53% of the C9:8- and the C11:10-propyl esters were lost by this and other side-reactions, whereas 74% of the C7:6-monomer was lost mainly through cyclisation.

![Diagram A](image1)

**Fig. 2.2:** Side-reactions of PHUE during transesterification with HCl-propanol. A) Cyclisation of propyl 3-hydroxy-6-heptenoate. B) Water addition to unsaturated side-chains (m = 1-3).

Improved results for the transesterification of mcl-PHAs were obtained with \( \text{BF}_3 \)-methanol as derivatisation reagent. PHO and PHUE were used to optimize the conditions of this reaction. Best results were obtained for a reaction time of 20 h at 80 °C with a \( \text{BF}_3 \)-concentration of 0.65 M. Similar to the transesterification with HCl-propanol, PHO was quantitatively methanolysed with \( \text{BF}_3 \)-methanol without forming side-products. After 8 h at 100 °C and 20 h at 80 °C the maximal recovery was reached as shown in Fig. 2.3. Further reaction time did not reduce the recovery substantially. showed a similar reactivity, but the conversion to side-products was
observed when the reaction time was further elongated, as shown in Fig. 2.4. At the maximum recovery at 80 °C only negligible peaks of side-products were observed in the chromatogram and about 98% of the polymer could be recovered as methyl esters.

The influence of the boron trifluoride concentration in the reaction mixture was studied for PHO and PHUE derivatised at 80 °C for 20 h. The optimum boron trifluoride concentration was 0.65 M for both polymers. Increasing this concentration to 0.87 M reduced the recovery of PHO from 100 to 53% (w/w) and the recovery of PHUE from 98 to 61% (w/w) (Fig. 2.5 and Fig. 2.6). Similarly, a decrease in the concentration of BF$_3$ to 0.16 M reduced the recovery of PHO to 34% (w/w) and the recovery of PHUE to 24% (w/w).

Low recoveries can also be caused by steps following the derivatisation of PHA. Jan et al. (Jan et al. 1995) described the loss of short 3-hydroxyalkanoic acid methyl esters during aqueous extraction, leading to incorrect results. To exclude this phenomenon for mcl-PHA, the aqueous phases of the extraction from PHO and PHUE were dried and analyzed, but no methyl esters could be detected. Hence, 3-hydroxyhexanoic methyl ester and the corresponding monomers with longer chains did not migrate to the aqueous phase.

To study the possible influence of the polymer concentration on the recovery of the BF$_3$-transesterification, the concentration of PHO was varied between 0.1 - 21.4 mg mL$^{-1}$. The recovered ratio for C8:0 to C6:0 was 8.9 (w/w) in all cases. However, the total recovery decreased slightly for small concentrations. For concentrations above ~3 mg mL$^{-1}$, the recovery was always greater than 97% (w/w), and was approximately (93.5 ± 1)% (w/w) in the concentration range 0.1 – 1.5 mg mL$^{-1}$. This is attributed to adsorptive effects of glass surfaces and of the GC injection system. Similar trends were observed for the BF$_3$-transesterification of PHU. This concentration was varied between 0.1 – 19.7 mg mL$^{-1}$. For example for C7:6, the mean value of recovery was 20% (w/w), with maximum deviations of +3.4% (w/w) for a concentration of 19.7 mg mL$^{-1}$, and -3.5% (w/w) for 0.2 mg mL$^{-1}$. 
**Fig. 2.3:** BF$_3$-methanol transesterification of PHO at 80 °C (●) and 100 °C (○). [BF$_3$] = 0.65 M. a) sum of monomers at 80 °C, b) sum of monomers at 100 °C, c) 3-hydroxyoctanoate at 80 °C, d) 3-hydroxyoctanoate at 100 °C, e) 3-hydroxyhexanoate at 100 °C, f) 3-hydroxyhexanoate at 80 °C. A measurement uncertainty of ± 4% was determined for 50 mg of PHA.

**Fig. 2.4:** BF$_3$-methanol transesterification of PHUE at 80 °C (●) and 100 °C (○). [BF$_3$]=0.65 M. a) sum of monomers at 80 °C, b) sum of monomers at 100 °C, c) 3-hydroxy-8-nonenoate at 80 °C, d) 3-hydroxy-8-nonenoate at 100 °C, e) 3-hydroxy-6-heptenoate at 80 °C, f) 3-hydroxy-10-undecenoate at 80 °C, g) 3-hydroxy-10-undecenoate at 100 °C, h) 3-hydroxy-6-heptenoate at 100 °C. A measurement uncertainty of ± 4% was determined for 50 mg of PHA.
Fig. 2.5: BF$_3$-methanol transesterification of PHO at 80 °C for 20 h with variable concentrations of BF$_3$. a) sum of monomers, b) 3-hydroxyoctanoate, c) 3-hydroxyhexanoate. A measurement uncertainty of ± 4% was determined for 50 mg of PHA.

Fig. 2.6: BF$_3$-methanol transesterification of PHUE at 80 °C for 20 h with variable concentrations of BF$_3$. a) sum of monomers, b) 3-hydroxy-8-nonenate, c) 3-hydroxy-10-undecenoate d) 3-hydroxy-6-heptenoate. A measurement uncertainty of ± 4% was determined for 50 mg of PHA.
The transesterification with BF$_3$-methanol was quantitative for all mcl-PHAs and the data were in good agreement with the results obtained by NMR as shown in Table 2.1. In contrast to mcl-PHAs, scl-PHAs were only recovered to 45-65% (w/w). This loss is due to the partitioning of short-chain methyl esters between the organic and the aqueous phase during extraction as described previously (Huijberts et al. 1994; Jan et al. 1995). In this work, NaCl-saturated water (Niskanen et al. 1978) was used instead of pure water, but this could not inhibit an extensive loss of short-chain methyl esters in our experiments. Hence, methanolysis should not be applied to scl-PHA when an aqueous extraction is necessary to remove reagents. In case of PHUE negligible amounts of side-products were observed. Compared with the HCl-propanolysis, more than three times the amount of C7:6-monomer, and increased amounts of C9:8 and C11:10 monomers were detected. The recovery improved from 55 to 98% (w/w).

The quantitative determination of PHA in biomass is of particular importance. Therefore, the transesterification method with BF$_3$-methanol was applied to biomass with different contents of PHOUE. Equal amounts (50 mg) of these biomass samples were methanolysed to study if other biomass components interfere with the methyl esters from PHA. A small number of weak additional peaks was observed, but they did not superpose the peaks of interest. The monomeric composition of PHOUE remained nearly constant for samples 1-4 as shown in Table 2.2. For instance, the relative amount of C8:0 in the polymer varied between 43.4% and 42.1% (w/w). Only in sample 5, larger deviations were observed.

To determine the PHA content alternatively, large samples of biomass (5 g) were extracted twice with methylene chloride and the polymer was precipitated with ethanol. The mass of PHOUE was determined by gravimetry after drying under vacuum. PHA contents between 3.4% and 16.8% (w/w) and between 2.9% and 15.9% (w/w) were determined by gravimetry and GC, as shown in Table 2.2. The correlation with the gravimetric determination showed a relative difference between 0% and 5.5% except at the lowest PHA concentration where the difference was 14.7% (Table 2.2). The extraction recovery was higher than the one determined by GC. This is reasonable since non-polar cell components like membrane lipids can be co-extracted and lead to an overestimation of the PHA content determined by gravimetry. Especially at low PHA contents this effect became marked. Further, we
observed that biomass with low contents of PHA comprises higher amounts of non-polar extractable impurities.

**Table 2.2**: Comparison between the quantitative analysis of mcl-PHA in biomass with GC after methanolysis\(^a\) and the gravimetric determination after extraction\(^b\). Five biomass samples\(^c\) with different contents of PHOUE were analyzed to determine the relative monomeric composition (% w/w) and the PHA content (% \(w_{\text{PHA}}/w_{\text{biomass}}\)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>C6:0 GC</th>
<th>C8:0 GC</th>
<th>C7:6 GC</th>
<th>C9:8 GC</th>
<th>C11:10 GC</th>
<th>PHA content GC</th>
<th>PHA content gravimetry</th>
<th>Relative difference PHA content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0</td>
<td>43.4</td>
<td>10.1</td>
<td>32.1</td>
<td>9.4</td>
<td>15.9</td>
<td>16.8</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>43.3</td>
<td>10.0</td>
<td>32.5</td>
<td>9.2</td>
<td>12.0</td>
<td>12.7</td>
<td>5.5</td>
</tr>
<tr>
<td>3</td>
<td>5.1</td>
<td>42.9</td>
<td>10.2</td>
<td>32.6</td>
<td>9.2</td>
<td>9.8</td>
<td>9.8</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>5.3</td>
<td>42.1</td>
<td>10.5</td>
<td>33.3</td>
<td>8.8</td>
<td>5.7</td>
<td>5.7</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>3.5</td>
<td>41.4</td>
<td>10.3</td>
<td>34.5</td>
<td>10.3</td>
<td>2.9</td>
<td>3.4</td>
<td>14.7</td>
</tr>
</tbody>
</table>

\(a\) 50 mg of biomass was methanolysed with BF\(_3\)-methanol (at 80 °C for 20 h). The measurement uncertainty is ≥ 7%.

\(b\) 5 g of biomass was extracted twice with methylene chloride, the solution was concentrated and precipitated with an excess of ethanol. The measurement uncertainty is around 5%.

\(c\) Sample 1 and 5 were produced by continuous fermentation under different conditions. Samples 2-4 were obtained by mixing sample 1 and 5 with varying ratios.

### 2.4 Conclusions

GC analysis is a common and efficient tool to quantify bacterial PHA. This study however emphasised the need for novel derivatisation methods for the analysis of mcl-PHA. A common transesterification method (Riis and Mai 1988) using HCl-propanol was shown to be inadequate for the quantitative analysis of mcl-PHA due to a serious underestimation of the PHA content. Although the adaptation of this method was successful for mcl-PHA with saturated side-chains, another derivatisation method was needed for mcl-PHA with unsaturated side-chains, to avoid an excessive formation of side-products. The main side-products of PHUE
derivatised with HCl-propanol were identified as the diastereomers of propyl 5-methyltetrahydrofuran-2-acetate formed by cyclisation of propyl 3-hydroxy-6-heptenoate, and as the products of water addition at the double bond. Other protic catalysts like sulphuric acid will produce the same side-products and hence generate incorrect results.

The formation of these side-products was avoided by using the Lewis-acid BF$_3$ in a water-free solvent. Transesterification with BF$_3$-methanol was applied successfully to all mcl-PHA investigated in this study. This novel method is well suited for the detection of mcl-PHA in biomass where other analytical methods fail. In comparison with the adapted HCl-propanol transesterification, only a few non-interfering additional peaks from other lipids of biomass appeared in the spectrum, which simplifies the analysis of data considerably.

NMR spectroscopy has been widely used to investigate various aspects of PHA, including copolymer compositions, linkage sequences or metabolic pathway studies. However, the measurement time for a single quantitative $^{13}$C spectrum was approximately 16 hours; therefore, NMR spectroscopy is not suitable for the routine determination of PHA copolymer composition. We also stirred whole cells in CDCl$_3$ for 5 hours and measured NMR spectra directly after filtration. In these spectra, several unidentified signals appeared that overlapped with PHA resonances. This means that the dissolution of PHA from biomass is not selective and that also the time-consuming extraction and purification steps can not be avoided for NMR analysis of PHA. This is in contrast to the GC method with BF$_3$-methanol presented in this work that was used to quantify PHA in biomass directly. This method is convenient to derivatise large amounts of samples in parallel and to measure them in a short time, since one GC-measurement requires only about 20 minutes.

2.5 Acknowledgment

We gratefully acknowledge the financial support by the Empa R&D fund. We also thank Thomas Ramsauer for assisting with polymer extractions, Elisabeth Michel for supporting GC analyses, and Matthias Nagel for chemical advices.
2.6 Supplementary information

Table 2.3: $^{13}$C NMR chemical shift assignments of the incorporated monomer units 3-hydroxypentanoate (C5:0), 3-hydroxyhexanoate (C6:0), 3-hydroxyoctanoate (C8:0), 3-hydroxy-6-heptenoate (C7:6), 3-hydroxy-8-nonenoate (C9:8), and 3-hydroxy-10-undecenoate (C11:10) in mcl-PHA.

<table>
<thead>
<tr>
<th>Position</th>
<th>C5:0</th>
<th>C6:0</th>
<th>C7:0</th>
<th>C8:0</th>
<th>C9:0</th>
<th>C11:0</th>
<th>C7:6</th>
<th>C9:8</th>
<th>C11:10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>168.8 – 169.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>38.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>69.8 – 71.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>26.7</td>
<td>35.9</td>
<td>33.5</td>
<td>33.6</td>
<td>33.8</td>
<td>33.8</td>
<td>32.9</td>
<td>33.8</td>
<td>33.8</td>
</tr>
<tr>
<td>5</td>
<td>9.3</td>
<td>18.3</td>
<td>27.1</td>
<td>24.7</td>
<td>25.0</td>
<td>25.1</td>
<td>29.2</td>
<td>24.5</td>
<td>25.0</td>
</tr>
<tr>
<td>6</td>
<td>13.8</td>
<td>22.4</td>
<td>31.5</td>
<td>29.0</td>
<td>29.5*</td>
<td>137.2</td>
<td>28.5</td>
<td>28.9*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>13.9</td>
<td>22.5</td>
<td>31.7</td>
<td>29.4*</td>
<td>115.3</td>
<td>33.5</td>
<td>29.2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>13.9</td>
<td>22.5</td>
<td>29.2*</td>
<td>138.5</td>
<td>28.8*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>14.0</td>
<td>31.8</td>
<td></td>
<td>114.6</td>
<td>33.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>22.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>139.0</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>14.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>114.2</td>
<td></td>
</tr>
</tbody>
</table>

* Positions not assigned

Fig. 2.7: Numbering of carbon atoms used for the signal assignments of compound 1.
### Table 2.4: Chemical shifts of the furanoyl isomers.

<table>
<thead>
<tr>
<th>Position a)</th>
<th>$\delta (^1H) / \text{ppm}$</th>
<th>$\delta (^{13}C) / \text{ppm}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1a</td>
<td>1b</td>
</tr>
<tr>
<td>1</td>
<td>1.21</td>
<td>1.23</td>
</tr>
<tr>
<td>2</td>
<td>4.11</td>
<td>3.97</td>
</tr>
<tr>
<td>3</td>
<td>1.49/2.05</td>
<td>1.48/1.99</td>
</tr>
<tr>
<td>4</td>
<td>1.61/2.16</td>
<td>1.63/2.06</td>
</tr>
<tr>
<td>5</td>
<td>4.40</td>
<td>4.22</td>
</tr>
<tr>
<td>6</td>
<td>2.43/2.61</td>
<td>2.46/2.64</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.05</td>
<td>4.05</td>
</tr>
<tr>
<td>9</td>
<td>1.65</td>
<td>1.65</td>
</tr>
<tr>
<td>10</td>
<td>0.94</td>
<td>0.94</td>
</tr>
</tbody>
</table>

a) For atom numbers, see Fig. 2.7.
Table 2.5: $^1$H, $^{13}$C-HMBC correlations used for NMR shift assignments and resolved $^1$H,$^1$H coupling constants.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HMBC correlations (w = weak)</th>
<th>$^1$J(H,H) / Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>H-(1) $\rightarrow$ C-(2, 3); H-(3) $\rightarrow$ C-(1, 4); H-(4) $\rightarrow$ C-(3, 6); H-(5) $\rightarrow$ C-(2, 3(w), 7(w)); H-(6) $\rightarrow$ C-(4, 5, 7); H-(8) $\rightarrow$ C-(7, 9, 10); H-(9) $\rightarrow$ C-(8, 10); H-(10) $\rightarrow$ C-(8, 9)</td>
<td>$^1$J(1,2) = 6.1; $^1$J(5,6a) = 6.6; $^1$J(5,6b) = 6.8; $^1$J(6a,6b) = 14.9; $^1$J(8,9) = 6.8; $^1$J(9,10) = 7.5</td>
</tr>
<tr>
<td>1b</td>
<td>H-(1) $\rightarrow$ C-(2, 3); H-(3) $\rightarrow$ C-(1, 4(w)); H-(4) $\rightarrow$ C-(3(w), 5(w)); H-(5) $\rightarrow$ C-(7(w)); H-(6) $\rightarrow$ C-(4, 5, 7); H-(8) $\rightarrow$ C-(7, 9, 10); H-(9) $\rightarrow$ C-(8, 10); H-(10) $\rightarrow$ C-(8, 9)</td>
<td>$^1$J(1,2) = 6.1; $^1$J(5,6a) = 6.7; $^1$J(5,6b) = 6.8; $^1$J(6a,6b) = 15.1; $^1$J(8,9) = 6.8; $^1$J(9,10) = 7.5</td>
</tr>
</tbody>
</table>

Fig. 2.8: Chemical structures with assignments of the absolute configuration of the mixture of isomers (1a and 1b) of (5-methyl-tetrahydro-furan-2-yl)-acetic acid propyl ester.

The signals at 4.40 and 4.22 ppm in the $^1$H NMR spectrum of the mixture of isomers (1a and 1b, respectively) do not interfere with other resonances. Relative amounts of 69% (1a) and 31% (1b) were determined from the integration of these signals. The relative configurations were established with a series of 1D NOESY spectra. When the pair of doublets at 1.2 ppm was selected, only H-(5) of 1a showed the proximity to the corresponding methyl group, together with H-(2) for both diastereomers at 4.11 and 3.97 ppm, respectively (see Fig. 2.9). Selective excitation
at 4.40 ppm (H-(5) of 1a) showed an NOE to H-(1), whereas the analogous experiment performed with H-(5) of 1b did not show an effect on the methyl group of this isomer (spectra not shown).

![Fig. 2.9: Sections of $^1$H NMR spectra of the 1a/1b mixture of diastereomers: a) 1D-NOESY experiment with selection of H-(1) at 1.2 ppm and b) normal $^1$H NMR spectrum.](image)

**Experimental**

The $^1$H and $^{13}$C NMR spectra were recorded at 400.13 (100.61) MHz on a Bruker Avance-400 NMR spectrometer. The 1D $^1$H and $^{13}$C NMR spectra and the $^1$H, $^1$H and $^1$H, $^{13}$C 2D correlation experiments were performed at 297 K using a 5 mm broadband inverse probe with z-gradient (100% gradient strength of 10 G cm$^{-1}$) and 90° pulse lengths of 6.7 µs ($^1$H) and 14.9 µs ($^{13}$C). The chemical shifts were referenced internally using the resonance signals of CDCl$_3$ at 7.26 ($^1$H) and at 77.0 ($^{13}$C) ppm. The gradient selected HSQC (Davis et al. 1992) (HMBC; (Bax and Marion 1988)) experiments were performed with selection of $^1$H, $^{13}$C coupling constants of 145 (10) Hz, gradient strengths of 80 : 20.1 : 11 : –5 (50 : 30 : 40.1), applying a carbon decoupling field of 3.1 kHz for the HSQC experiments (GARP decoupling; (Shaka et al. 1985)). Before processing the data matrices of 1024 x 512 were zero filled to 1024 x 1024. The HSQC-TOCSY (Palmer et al. 1992) spectra were recorded with the selection of $^1$J ($^1$H,$^{13}$C) = 145 Hz and a 27 µs 90° pulse length for the TOCSY transfer with a total mixing time of 110 ms, applying the above mentioned carbon decoupling
conditions, data matrices and processing conditions. The $^1\text{H},^1\text{H}$ connectivity’s and the relative configuration of the isomers were established by DQF-COSY and by 1D NOESY experiments (mixing time of 1s, 50 ms selective 180° inversion pulse with Gaussian shape) (Stott et al. 1997).
Chapter 3

Biosynthesis of medium-chain-length poly(3-hydroxyalkanoate): endotoxin reduction by phosphorus limitation
3.1 Introduction

PHAs with side chains of medium-chain-length (mcl-PHA, monomers with 6-14 carbon atoms) possess a wide range of physical and chemical properties due to their manifold monomeric compositions. They are synthesized by microorganisms under appropriate growth conditions, such as nitrogen limitation (Witholt and Kessler 1999). The production of mcl-PHAs is well established with Gram-negative bacteria but its contamination with lipopolysaccharide (LPS) from the outer membrane restrains the application in the medical field (Valappil et al. 2007; Williams et al. 1999). LPS, also referred to as endotoxin, represents one of the microbial molecular signals responsible for the activation of the innate immune system (Trent et al. 2006). It is recognised by receptor TLR4 (toll-like receptor 4) that is present on many cell types like macrophages and dendritic cells. Even nanogram amounts of LPS cause fever, septic shock and other immune responses when injected intravenously into humans. Hence, its concentration in medical products has to be very low, e.g. the endotoxic activity has to be smaller than 20 endotoxic units (EU) per medical device (U.S. Department of health and human services 1997).

To obtain mcl-PHA of medical purity, several purification steps are necessary for its recovery from biomass and special procedures have to be applied for endotoxin removal (Lee et al. 1999; Williams et al. 2001). This complicates the downstream processing and increases the production costs. Preventing the synthesis of LPS or reducing their endotoxic activity during the production of mcl-PHA would drastically simplify the recovery procedure. However, LPS are essential for the survival of Gram-negative bacteria as they augment the rigidity of the cell wall and protect bacteria for instance from bile salts, gut enzymes and antibiotics (Holst 1995). Among Gram-negative bacteria, only mutants of Neisseria meningitidis were viable without LPS, presumably due to the presence of capsular polysaccharide (Bos and Tommassen 2005; Dixon and Darveau 2005; Raetz et al. 2007).

Indeed, the biological activity of LPS and in particular its endotoxic activity is largely dependent on the structure of lipid A, the lipophilic part of LPS (Dixon and Darveau 2005; Trent et al. 2006). Reducing the degree of acylation or phosphorylation drastically decreases the biological activity of LPS as shown by Rietschel et al. (Fig. 3.1, (Rietschel et al. 1994)). Certain bacteria adapt the structure of lipid A to the environmental growth conditions. Yersinia pestis for instance reduces the degree of
acylation of lipid A during invasion of mammals to avoid the host’s immune defence (Knirel et al. 2005). Furthermore, it has been observed that some pathogenic organisms lack one or both phosphate groups of lipid A (Trent et al. 2006). But so far, it has been unknown whether non-pathogenic bacteria can be stimulated by environmental conditions to reduce the degree of phosphorylation of lipid A.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Reduction factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>$10^2$</td>
</tr>
<tr>
<td>1b</td>
<td>$10^2$</td>
</tr>
<tr>
<td>1a + 1b</td>
<td>Non toxic</td>
</tr>
<tr>
<td>2</td>
<td>$10^7$</td>
</tr>
<tr>
<td>3a</td>
<td>$10^2$</td>
</tr>
<tr>
<td>3a + 3b</td>
<td>$10^7$</td>
</tr>
<tr>
<td>4</td>
<td>$10^2$</td>
</tr>
<tr>
<td>5</td>
<td>$10^1$</td>
</tr>
</tbody>
</table>

In this work we investigated whether the endotoxic activity of crude PHA can be lowered by phosphate-limited growth conditions such that the phosphorylation of lipid A is reduced. This is of great importance because the endotoxic activity of lipid A is decreased by a factor of 100 or more when one phosphate group is removed (Rietschel et al. 1994). To realize such a scenario, we used carbon and nitrogen limited conditions ((C,N)-limitation) as starting point for the cultivation of *Pseudomonas putida* GPo1 to induce PHA accumulation (Hartmann et al. 2006). Next, the phosphate concentration in the mineral medium was reduced stepwise to reach phosphorus limitation (P-limitation) as part of a triple (C,N,P)-limitation. Drastic changes in the cell composition were observed with P-limitation.
3.2 Materials and methods

Biosynthesis of mcl-PHA

Polyhydroxyoctanoate (PHO) consisting of 87% (w/w) 3-hydroxyoctanoate and 13% (w/w) 3-hydroxyhexanoate was produced by balance-controlled continuous cultivation of P. putida GPo1 (ATCC 29347) in a 3 L bioreactor (KLF 2000, Bioengineering, Wald, Switzerland) with a working volume of 2.6 L and a dilution rate (D) of 0.125 h⁻¹. The preculture was prepared as described elsewhere (Durner et al. 2000; Hartmann et al. 2006). An aliquot of 200 mL of a shake-flask culture was used to inoculate the bioreactor containing 2.4 L of fermentation medium.

For continuous cultivation, the following medium was used (per liter): 0.80 g of EDTA-Na₂·2H₂O, 3.78 g of (NH₄)₂SO₄, 1.00 g of MgSO₄·7H₂O, 0.28 g of FeSO₄·7H₂O, 0.03 g of CaCl₂·2H₂O and 1.05 g of MOPS was added as pH buffer. The carbon to phosphorus ratio (C/P) in the mineral medium was adjusted by the addition of KH₂PO₄ to the medium (e.g. 1.17 g of KH₂PO₄ for C/P = 30 g g⁻¹), whereas the carbon source octanoic acid was pumped separately to the culture broth (resulting in a carbon feed concentration of 8.0 g C L⁻¹). As a consequence the carbon to nitrogen ratio (C/N) in the medium feed was fixed to 10 g g⁻¹. For fermentations with nitrogen excess the concentration of (NH₄)₂SO₄ was increased to 11.34 g L⁻¹ resulting in a C/N of 3.3 g g⁻¹. Furthermore, 1 L of the medium was supplemented with 2 mL of trace element stock solution that contained per liter: 12.22 g of MnCl₂·4H₂O, 1.27 g of CoCl₂·6H₂O, 2.00 g of CuCl₂·2H₂O, 7.50 g of ZnSO₄·7H₂O, and 0.5 g of Na₂MoO₄·2H₂O in 1M HCl. The medium was sterilized by filtration. Octanoic acid (95 % w/w) was pumped directly into the culture vessel by using a dosimat (Metrohm, Herisau, Switzerland). The dissolved oxygen concentration was maintained between 30 and 50% relative to the maximum oxygen concentration in the aerated culture medium before inoculation. Poly(propylene glycol) (Mₘ = 4000) was added to octanoic acid to 4% (v/v) to avoid excessive foam formation. For each phosphate concentration, at least five volume exchanges were allowed to reach steady state. Two measurements were made within 24 h.

The fermentation broth was concentrated by centrifugation at 4'500 x g for 20 min. The paste was lyophilized for 48 h, ground, and the dry biomass was stored in a closed container at -25 °C. The supernatant was filtrated (PES filter, 0.22 µm) and stored at -25 °C.
Analytical methods

**PHA determination.** The PHA content in biomass was determined in duplicate by GC after propanolysis of biomass as described in Chapter 2.

**Endotoxin detection.** The endotoxic activity in aqueous solutions was determined with the PyroGene recombinant factor C assay (Lonza Bioscience, Visp, Switzerland). For the endotoxic activity of biomass, 1 g of dried biomass was washed twice with 50 mL of 0.9% NaCl by vortexing and centrifugation to remove free LPS. The pellet was freeze dried. Twenty mg washed biomass was suspended in 10 mL buffer (2.3 g SDS, 11.5 mL glycerol, 12.4 mL 0.5 M Tris-HCl and 76.1 mL H₂O adjusted to pH = 6.8 with HCl), heated up to 95 °C for 10 min to lyse the cells and then shaken at 150 rpm at room for 24 h. The suspension was filtrated (PES filter, 0.22 µm) and diluted in three steps (1:100, 1:100 and 1:500) to a total dilution of 1:5x10⁶. One hundred µl of this solution were used to measure the endotoxic activity with the recombinant factor C assay. For the endotoxic activity of PHA, 200 mg of washed biomass was extracted with 5 mL of methylene chloride under stirring at room temperature for 20 h. The suspension was centrifuged, filtrated (Nylon filter, 0.45 µm) and 3.5 mL of the resulting polymer solution was poured into a small Petri dish (d=4 cm) that had been depyrogenized previously at 200 °C for 6 h. The solvent was slowly evaporated (3 days) to form PHA films with a homogenous surface. Endotoxins were extracted from the surface with 3 mL of endotoxin-free water at room temperature for 24 h. An aliquot of 100 µl of this solution was then used to measure the endotoxic activity.

The recombinant factor C of the Pyrogen assay is activated by endotoxin-binding, and the active moiety created then acts to cleave a synthetic substrate, which results in the generation of a fluorogenic compound. After one hour of incubation, the fluorescence at 440 nm can be measured by exciting at 380 nm and the endotoxic activity is determined relative to an endotoxin standard. One endotoxic unit (EU) corresponds to about 0.1 ng of standard endotoxin.

**Biomass concentration.** Cells were collected on pre-weighed polycarbonate filters (0.2 µm, Nucleopore, Sterico AG, Dietikon, Switzerland). The filters were first washed with 10 mM MgCl₂, dried overnight at 105 °C and weighed. An aliquot of 3 mL of cell suspension was filtered through the filter and the filter was dried again at 105 °C overnight. The mass difference was used to calculate the concentration of dry biomass in the culture.
Optical density. The cell suspension was diluted 1:100 with deionised water and the optical density was determined at 450 nm.

Analyses of substrate concentrations in the culture supernatant. Ammonium was measured by using a photometric ammonium test (Spectroquant, Merck Darmstadt, Germany). The measurement range was linear between 0.01 and 3 mg N L\(^{-1}\) (present as ammonium). If necessary, the samples were diluted with deionised water.

Octanoic acid was measured by gas chromatography after derivatisation to propyl esters. An aliquot of 5 mL of methylene chloride containing 1 mg mL\(^{-1}\) of 2-ethyl-2-hydroxybutyric acid as internal standard was added to 2 mL of supernatant in a 20 mL Pyrex tube. Five mL of a 20/80 (v/v) mixture of HCl (37%) and \(n\)-propanol was added, the tube was tightly sealed, and the mixture was vigorously shaken. The tube was placed in an oven at 80 °C for 16 h. The subsequent steps were similar to the derivatisation of PHA as described above.

Phosphate was determined according to the ascorbic acid molybdane blue method (Chen et al. 1956). An aliquot of 10 mL of supernatant was mixed with 5 mL of freshly prepared reagent (containing sulphuric acid, ammonium molybdate and ascorbic acid) and supplemented with deionised water to 50 mL. The mixture was shaken and the absorbance at 800 nm was measured after 15 minutes of reaction at room temperature. The measurement range was linear between 0.1 and 100 mg P L\(^{-1}\) (present as phosphate), for higher concentrations dilution was necessary.

Analyses of biomass. Total phosphorus was determined as follows: About 300 mg of dried biomass and 3 mL of HNO\(_3\) (65%) were added to a 20 mL cone. An aliquot of 2 mL of distilled water and 1 mL of H\(_2\)O\(_2\) (30%) were added to a 30 mL vessel and the cone was placed therein. The sample was digested in a microwave digester (Milestone, MLS 1200 Mega, Sorisole, Italy) for 15 minutes with a stepwise increase from 100 to 500 W. After cooling, the clear solution was filled up with distilled water to a volume of 10 mL. The solution was diluted between 1:10 and 1:100 with 2% HNO\(_3\) and the emission was measured with ICP-OES (Perkin Elmer, Optima 3000, Schwerzenbach, Switzerland) at 213.6 and 214.9 nm. Standard solutions were prepared from a 1.00 mg P L\(^{-1}\) standard solution (Alfa Aesar, Karlsruhe, Germany) for external calibration.

Phospholipids were extracted with chloroform-methanol according to method of Folch (Folch et al. 1957). Quantitative analysis was done by a colorimetric method based on the formation of a complex between phospholipids and ammonium
ferrothiocyanate (Stewart 1980). Phospholipids extracted from 200 mg of dried biomass were dissolved in 2 mL of chloroform. To an aliquot of 0.1 mL, 1.9 mL of chloroform and 2 mL of thiocyanate reagent was added. The mixture was vortexed for 1 min and centrifuged for 10 min at 2'000 x g. The lower layer was removed and the light absorbance of this solution was measured at 488 nm. 1,2-Dipalmitoyl-

\[ \text{glycero-3-phosphoethanolamine} \]

was used for calibration. The linear range was between 1.5 and 30 mg L\(^{-1}\).

### 3.3 Results and discussion

#### General observations

The adaptation of *P. putida* GPO1 to P-limitation was generally slow and a drastic decrease of the phosphorus concentration in the mineral medium feed (e.g. from 200 to 100 mg P L\(^{-1}\)) was not tolerated by the cells. Excessive foam generation as well as biofilm formation were observed that soon led to unstable process conditions. Even with small changes of the phosphorus concentration, wall growth could not be prevented completely. C/P ratios higher than 90 g g\(^{-1}\) lead to unstable cultures. One possible reason is that the specific growth rate of the cells was decreased by very low phosphorus concentrations, so that octanoic acid could not be consumed completely, resulting in a substrate inhibition finally leading to a wash-out of the culture. Under phosphorus limitation, the cell shape became more rod-like and the brown colour of the fermentation broth brightened.

#### Nutrient limitations

The accumulation of PHA in bacteria can be triggered by nutrient limitations such as nitrogen limitation. Carbon limitation is commonly applied to prevent substrate inhibition. For the production of mcl-PHA with *P. putida* GPO1 the combined (C, N)-limitation strategy has successfully been applied (Durner et al. 2000; Hartmann et al. 2006; Ruth et al. 2007).

Nutrient limitation has often been described using the kinetic model of Monod (Monod 1949), although this model neglects bacterial adaptations to prolonged nutrient limitation (Ferenci 1999). The concentration of the limiting nutrient (s) in the culture broth can be calculated with Monod’s equation if the specific growth rate (\(\mu\)), the maximum specific growth rate (\(\mu_m\)) and the half saturation coefficient (\(K_s\)) are known:
For chemostat cultures at steady state the specific growth rate is equal to the dilution rate and consequently the concentration of the limiting nutrient is constant. However, the Monod model is only valid for single nutrient limitation. A simple kinetic model for dual and triple-limited fermentations does not exist. Hence a simple approximation was used to define kinetic nutrient limitation. Here limitation was defined as the state where the residual concentration of a nutrient becomes virtually constant at a low level. A further decrease of the nutrient concentration in the medium does not affect the residual concentration significantly.

The measured concentration of a nutrient is in fact compromised by method of measurement. During the time between sample drawing and centrifugation, the bacteria continue to consume the residual nutrients in the medium. The measured nutrient concentrations are consequently lower than at the time point of sampling. Extrapolating from the observed nutrient consumption rates, a decrease in phosphorus concentration of about 0.7 mg L\(^{-1}\) has to be considered for a concentration of about 7 g biomass L\(^{-1}\) for a delay of 2 minutes between sampling and centrifugation. Similarly, a reduction of 34 mg/L has to be taken into account for carbon and 3.4 mg/L for nitrogen for a period of 2 minutes.

The limiting (measured) concentrations of nutrients obtained were 0.3 mg P L\(^{-1}\), 3 mg N L\(^{-1}\), and 10 mg C L\(^{-1}\). The values for phosphorus and nitrogen appeared reasonable as similar values, 0.2 mg P/L and 2.2 mg N/L could be calculated with \(K_s\) values from \(E.\ coli\) and \(P.\ putida\) PGA1 (17 µM for phosphate and 460 µM for ammonium, respectively) using the Monod equation (Annuar et al. 2007; Owens and Legan 1987). According to the experiments, phosphorus limitation started at a C/P = 65 g g\(^{-1}\). Further, it was observed that nitrogen became non-limiting at a C/P = 75 g g\(^{-1}\) and octanoic acid at a C/P = 85 g g\(^{-1}\). According to these boundaries four regimes of nutrient limitation could be distinguished:

- Dual (C,N)-limited regime: \(C/P = 30-65\) g g\(^{-1}\) (I)
- Triple (C,N,P)-limited regime: \(C/P = 65-75\) g g\(^{-1}\) (II)
- Dual (C,P)-limited regime: \(C/P = 75-85\) g g\(^{-1}\) (III)
- Single P-limited regime: \(C/P = 85-95\) g g\(^{-1}\) (IV)

\[ s = K_s \frac{\mu}{\mu_m - \mu} \]
Fig. 3.2: Residual concentration of nutrients in the supernatant and their original concentration in the medium of fermentation 1. A) Free carbon from octanoic acid in medium (◆), free carbon from octanoic acid in supernatant (◇), free nitrogen (present as NH$_4^+$) in medium (●), free nitrogen (present as NH$_4^+$) in supernatant (O). B) Free phosphorus (present as phosphate) in medium (■), free phosphorus (present as phosphate) in supernatant (□), bound phosphorus in supernatant (▲). Limitation regimes: I) (C,N)-limited, II) (C,N,P)-limited, III) (C,P)-limited, IV) (P)-limited.

Interestingly up to 6 mg P L$^{-1}$ were found in extracellular substances under P-limiting conditions (Fig. 3.2B). Likewise, in the N-limited regime (C/P = 30-70 g g$^{-1}$) a substantial amount of nitrogen was found as extracellular protein (140-200 mg protein L$^{-1}$, corresponding to ~24-34 mg N L$^{-1}$). Hence, the quantity of extracellular substances containing nitrogenous compounds was non-negligible. More detailed analyses to identify the compounds were not carried out.
Biomass production and PHA accumulation

The optical density (OD) and the cell dry weight (CDW) increased towards higher C/P and reached a maximum at C/P = 80 g g\(^{-1}\) (Fig. 3.3A). This increase was mainly due to enhanced PHA accumulation as the concentration of residual biomass remained nearly constant until C/P = 70 g g\(^{-1}\) and only slightly decreased towards higher C/P. The PHA content of dried biomass increased with P-limitation (C/P > 60 g g\(^{-1}\)) from 10 w% to 25 w% from regime I to regime III (Fig. 3.3B).

Phosphorus and phospholipid in biomass

The phosphorus content in residual biomass and the content of phospholipids decreased with P-limitation (Fig. 3.4). Phosphorus in residual biomass was reduced under P-limited conditions to virtually 50% of the maximum value, whereas the content...
in phospholipids was decreased to 10% of its maximum value. We therefore could confirm that membrane phospholipids play a key role in the phosphorus pool of *P. putida* GPo1, as has been observed for *P. diminuta*, *P. aeruginosa* and *P. fluorescens* (Minnikin and Abdolrah.H 1974; Minnikin et al. 1974; Yuasa 2002). For these species, most of the membrane phospholipids were substituted under strong P-limitation by nitrogen or sulphur containing lipids (Minnikin and Abdolrah.H 1974; Minnikin et al. 1974; Yuasa 2002). Interestingly, under conditions of excess phosphate (C/P = 30 and 40 g g\(^{-1}\)) the phospholipid content was also decreased relative to the observed maximum at C/P of 50 g g\(^{-1}\). This may be due to a shift in the phospholipid composition with excess phosphate eventually causing an underestimation.

![Graph showing the content of phosphorus and phospholipids in residual biomass as a function of C/P ratio](image)

**Fig. 3.4:** Content of phosphorus (◊) and phospholipids (●) in residual biomass of fermentation 1. Limitation regimes: I) (C,N)-limited, II) (C,N,P)-limited, III) (C,P)-limited, IV) (P)-limited.
Endotoxins in biomass and extracted PHA

The endotoxic activity of biomass was considerably reduced with P-limitation, as shown in Fig. 3.5A. Because the measurement of endotoxic activities suffered from a large uncertainty, the fermentation experiment was repeated twice for independent measurements. Large variations were observed between the three independent experiments, but the same trend was clearly found for each experiment – the endotoxic activity dropped between 12- and 20-fold. One reason for these variations might be LPS that were released from the cell membrane, partially adhering to biomass during centrifugation. It has been observed that special fermentation conditions trigger the release of endotoxins (Svensson et al. 2005). Biomass was washed twice during work-up to remove free LPS, but this procedure might not have been quantitative. For fermentation 1 (F1) the endotoxic activity of biomass dropped from $1.46 \times 10^6$ (C/P = 30 g g$^{-1}$) to $1.22 \times 10^5$ EU mg$^{-1}$ biomass (C/P = 90 g g$^{-1}$). Fermentation 2 (F2) and 3 (F3) showed endotoxic activities of $2.35 \times 10^6$ and $3.55 \times 10^6$ EU mg$^{-1}$ biomass (C/P = 40 g g$^{-1}$) and $1.04 \times 10^5$ and $2.62 \times 10^5$ EU mg$^{-1}$ biomass (C/P = 90 g g$^{-1}$), respectively. An analogous trend was measured for PHA extracted from biomass although here the effect was more pronounced. Methylene chloride was chosen as a standard solvent because it is suitable for the extraction of all mcl-PHAs and is often used for this purpose. Again the endotoxic activities of PHA from three independent fermentation experiments varied considerably, but showed the same trend (Fig. 3.5B). In particular for the regime III and IV PHA showed a very low endotoxic activity of 18, 66 and 28 EU g$^{-1}$ PHA for F1, F2, and F3, respectively. In general, the endotoxic activity of PHA was considerably reduced, e.g. by a factor of about 300 from C/P = 30 g g$^{-1}$ to CP = 90 g g$^{-1}$ for F1, whereas the endotoxic activity of biomass was reduced only by a factor of 12 for F1.

The reason for the decreased endotoxic activity of biomass and of PHA produced under phosphorus limitation can be explained either by structural changes of lipid A, most apparently from biphosphorylated lipid A to the mono- or non-phosphorylated form (compare Fig. 3.1), or by a reduction of the concentration of LPS in the outer membrane. Although the latter is rather unlikely because of the necessity for membrane stabilisation and protection, we have so far been unsuccessful in confirming the structural change of lipid A by separation of lipid A from other cell components with thin layer chromatography and analysis by mass spectrometry. Thus, more detailed investigations are required for clarification.
Fig. 3.5: A) Endotoxic activity of dried biomass B) Endotoxic activity of PHA extracted from dried biomass with methylene chloride. Results were obtained from three independent fermentation experiments. Fermentation 1 (■), fermentation 2 (●) and fermentation 3 (▲). Limitation regimes: I) (C,N)-limited, II) (C,N,P)-limited, III) (C,P)-limited, IV) (P)-limited.

The consequences of P-limitation seem to be similar for phospholipids and LPS. In contrast to phospholipids, the reduction of the endotoxic activity started already when the concentration of free phosphorus was low (0.3-5 mg P L$^{-1}$) but not limiting and continued with P-limitation.

Yield coefficients
Carbon and nitrogen yield coefficients for residual (PHA-free) biomass were hardly affected by P-limitation (Table 3.1). The carbon yield coefficient varied between 0.69 and 0.81 g g$^{-1}$ and the nitrogen yield coefficient between 7.60 and 8.27 g g$^{-1}$ for F1. The phosphorus yield coefficient rose from 30 to 62 g g$^{-1}$ for a change of C/P from 30
to 90 g g\(^{-1}\) for F1. F2 and F3 showed similar results. The carbon yield coefficients for F2 and F3 varied only slightly as well as the nitrogen yield coefficients. The phosphorus yield coefficients increased by about 100% for a change of C/P from 40 to 90 g g\(^{-1}\) (F2 and F3).

By using the modified stoichiometric approach (Egli 1991), dual and triple-limited growth regimes can be estimated. According to this approach growth limiting nutrients are completely converted into biomass under nutrient limitation. The ratio of these nutrient concentrations in the medium feed (C\(_{f}\), N\(_{f}\), and P\(_{f}\), respectively) equals the inverse ratio of the corresponding growth yield coefficients (Y\(_{X/C}\), Y\(_{X/N}\), and Y\(_{X/P}\), respectively). The growth yield coefficients for total biomass can be replaced in this calculation by growth yield coefficients for residual biomass since this leads to the same result. Under triple (C,N,P)-limitation the following equations must hold:

\[
\frac{C_{f}}{P_{f}} \approx \frac{Y_{X/P}^{C,N,P-\text{lim.}}}{Y_{X/C}^{C,N,P-\text{lim.}}} \tag{2}
\]

\[
\frac{N_{f}}{P_{f}} \approx \frac{Y_{X/P}^{C,N,P-\text{lim.}}}{Y_{X/N}^{C,N,P-\text{lim.}}} \tag{3}
\]

The yield ratios of F1 are shown in Table 3.1. According to the stoichiometric approach, triple limitation had to be found between C/P = 40 and 70 g g\(^{-1}\). For F2 and F3 triple limitation was reached from C/P = 50 to 70 g g\(^{-1}\). This estimation was in clear contrast to the previous determination of nutrient-limited growth regimes based on the kinetic approach, where the triple-limited regime was determined from C/P 65 to 75 g g\(^{-1}\). The stoichiometric P-limitation was reflected in the decreasing phosphorus content in biomass (Fig. 3.4). P-limitation according to the kinetic approach went together with a considerable increase of the PHA content in biomass. This was similar to N-limitation, where higher C/N ratios enhance PHA accumulation (Durner et al. 2000).

Hence, the stoichiometric approach indicated physiological adaptations to reduced concentrations of phosphorus. Effective limitation in the sense of very low residual phosphorus concentrations in the supernatant and enhanced PHA accumulation was only determined with the kinetic approach.
Table 3.1: Yield factors for residual biomass and yield ratios for the determination of dual and triple-limited growth regimes of fermentation 1. Dual limitation is reached when the yield ratio equals the ratio of the corresponding nutrients in the feed medium (C/P or N/P). For triple limitation both yield ratios have to fulfil this condition.

<table>
<thead>
<tr>
<th>Regime</th>
<th>C/P [g g(^{-1})]</th>
<th>N/P [g g(^{-1})]</th>
<th>(Y_{X/C} [g g^{-1}])</th>
<th>(Y_{X/P} [g g^{-1}])</th>
<th>(Y_{X/N} [g g^{-1}])</th>
<th>(Y_{X/P}/Y_{X/C} [g^2 g^{-2}])</th>
<th>(Y_{X/P}/Y_{X/N} [g^2 g^{-2}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>30</td>
<td>3.0</td>
<td>0.79</td>
<td>30.1</td>
<td>7.93</td>
<td>38.0</td>
<td>3.8</td>
</tr>
<tr>
<td>I</td>
<td>40</td>
<td>4.0</td>
<td>0.77</td>
<td>31.9</td>
<td>7.73</td>
<td>41.2</td>
<td>4.1</td>
</tr>
<tr>
<td>I</td>
<td>50</td>
<td>5.0</td>
<td>0.76</td>
<td>39.2</td>
<td>7.60</td>
<td>51.6</td>
<td>5.1</td>
</tr>
<tr>
<td>I</td>
<td>60</td>
<td>6.0</td>
<td>0.81</td>
<td>48.8</td>
<td>8.11</td>
<td>60.3</td>
<td>6.0</td>
</tr>
<tr>
<td>II</td>
<td>70</td>
<td>7.0</td>
<td>0.80</td>
<td>57.6</td>
<td>7.99</td>
<td>72.1</td>
<td>7.2</td>
</tr>
<tr>
<td>III</td>
<td>80</td>
<td>8.0</td>
<td>0.76</td>
<td>62.1</td>
<td>8.07</td>
<td>81.7</td>
<td>7.7</td>
</tr>
<tr>
<td>IV</td>
<td>90</td>
<td>9.0</td>
<td>0.69</td>
<td>62.0</td>
<td>8.27</td>
<td>90.5</td>
<td>7.5</td>
</tr>
</tbody>
</table>

\(Y_{X/Y}\): growth yield factor for residual biomass, \(Y_{X/Y}/Y_{X/Z}\): yield ratio.

Cell physiological aspects with different nutrient limitations

Nitrogen limitation was applied in the previous experiments combined with C- and or P-limitation because it was considered to be essential for PHA accumulation. To see if the N-limitation played a crucial role for the PHA accumulation and for the endotoxic activity, the concentration of nitrogen in the mineral medium was increased threefold (resulting in a C/N = 3.3 g g\(^{-1}\)) to ensure excess nitrogen. Two steady states, C/P = 60 and C/P= 80 g g\(^{-1}\), were analysed and compared with the results obtained with C/N = 10 g g\(^{-1}\) (see Table 3.2).

Excess nitrogen (C/N = 3.3 g g\(^{-1}\)) induced an enhanced production of biomass of 8.3 g L\(^{-1}\) and an increased consumption of phosphorus causing P-limitation for C/P = 60 g g\(^{-1}\). PHA accumulation in dried biomass was similar to the one under N-limited conditions. The endotoxic activity of PHA and biomass, however, was about 2.5
times higher under nitrogen in excess than under nitrogen limitation. Interestingly, the content of phospholipids was reduced by more than 50% with excess nitrogen.

Excess carbon (30 mg L\(^{-1}\)) and excess nitrogen (1'740 mg L\(^{-1}\)) was measured in the supernatant for C/P = 80 g g\(^{-1}\) and C/N = 3.3 g g\(^{-1}\) and hence this steady state was only P-limited. The cell dry weight was reduced, the phospholipid content strongly reduced and the PHA content was increased by about 50%. In comparison, for a C/P = 80 g g\(^{-1}\) and a C/N = 3.3 g g\(^{-1}\), significant amounts of free nitrogen (48 mg L\(^{-1}\)) could be measured in the supernatant and hence this growth state was (C,P)- but not N-limited. Concomitantly PHA accumulation was more efficient at a C/N = 10 g g\(^{-1}\). Significant differences were observed for the endotoxic activity of biomass and PHA which was about 8- respectively 126-times smaller with C/N = 10 g g\(^{-1}\) compared to the fermentation with excess nitrogen (C/N = 30 g g\(^{-1}\)).

Concluding, increasing C/P from 60 to 80 g g\(^{-1}\) did raise the endotoxic activity with C/N = 3.3 g g\(^{-1}\) to some extent. Thus, PHA accumulation was higher and the activity of endotoxins was lower with a high carbon to nitrogen ratio. Hence, a low N/P ratio (6 and 8 g g\(^{-1}\)) was favourable over high N/P values (18 and 24 g g\(^{-1}\)) for P-limited conditions.

These effects can be explained by the reduced flexibility of cells with additional nitrogen limitation, compared to a state with nitrogen in large excess, forcing the bacteria to efficiently use phosphorus and to accumulate mcl-PHA from carbon feed. For instance the synthesis of proteins and membrane lipids lacking phosphorus (e.g. ornithine-based lipids) will be facilitated with excess nitrogen compared to N-limited conditions. One could argue that both fermentations with C/P = 80 g g\(^{-1}\) were non-nitrogen limited and should give similar results. But it seems that a large nitrogen excess has an inhibiting effect on the cell growth at this C/P ratio.

Some wall growth leading to biofilm formation was observed with P-limitation independent from N-limitation. It has been observed for \textit{P. putida} in a laminar flow cell reactor that biofilm accumulation rate was highest at C/P =100 g g\(^{-1}\) (Rochex and Lebeault 2007). This confirms our observation that biofilm formation increased with a higher C/P. Calcium crosslinking with extracellular polysaccharides is supposed to be important for the matrix of biofilms and can be inhibited by increased precipitation of calcium phosphate (Turakhia and Characklis 1989). But this is only possible with excess phosphate and hence biofilm formation seems to be an intrinsic problem of P-limitation.
Table 3.2: Comparison of different nutrient limitations with C/N = 10 and C/N = 3.3 g g⁻¹.

<table>
<thead>
<tr>
<th>Limited nutrients</th>
<th>C/P</th>
<th>C/N</th>
<th>OD</th>
<th>CDW</th>
<th>CDW-PHA</th>
<th>PHA</th>
<th>Phospholipid content</th>
<th>Endotoxins</th>
<th>Endotoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[g g⁻¹]</td>
<td>[g g⁻¹]</td>
<td>[-]</td>
<td>[g L⁻¹]</td>
<td>[g L⁻¹]</td>
<td>[%]</td>
<td>[%]</td>
<td>EU mg⁻¹ biomass</td>
<td>EU g⁻¹ PHA</td>
</tr>
<tr>
<td>C, N</td>
<td>60</td>
<td>10</td>
<td>25.6</td>
<td>7.3</td>
<td>6.5</td>
<td>10.7</td>
<td>4.4</td>
<td>4.2 x 10⁵</td>
<td>828</td>
</tr>
<tr>
<td>C, P</td>
<td>60</td>
<td>3.3</td>
<td>26.6</td>
<td>8.3</td>
<td>7.3</td>
<td>11.5</td>
<td>2.0</td>
<td>10.9 x 10⁵</td>
<td>2208</td>
</tr>
<tr>
<td>C, P</td>
<td>80</td>
<td>10</td>
<td>31.7</td>
<td>8.2</td>
<td>6.1</td>
<td>25.6</td>
<td>1.0</td>
<td>2.2 x 10⁵</td>
<td>21</td>
</tr>
<tr>
<td>P</td>
<td>80</td>
<td>3.3</td>
<td>26.9</td>
<td>7.5</td>
<td>6.2</td>
<td>16.5</td>
<td>1.0</td>
<td>17.6 x 10⁵</td>
<td>2661</td>
</tr>
</tbody>
</table>

OD: optical density measured at 450nm, CDW: cell dry weight, CDW-PHA: residual biomass.

3.4 Conclusions

*P. putida* GPo1 cultivated continuously under P-limitation combined with carbon and nitrogen limitation drastically changed its cell composition. The amount of phospholipids in dried biomass was decreased by about 90% under P-limitation compared to a state without P-limitation. In parallel, the endotoxic activity of biomass was reduced to 10% of its initial value. Furthermore, P-limitation combined with N-limitation considerably increased the PHA accumulation. With combined (C,P)-limitation and excess nitrogen the PHA accumulation was significantly lower and the endotoxic activity was notably increased. This clearly demonstrated that combined (C,N,P)-limitation leads to a better performance of the culture than (C,P)-limitation with nitrogen excess.

Consequently, by selecting appropriate fermentation conditions, the contamination of PHA with endotoxins can be significantly reduced. However, P-limitation cannot be extended arbitrarily as the cell growth rate becomes restricted. Further, P-limitation of *P. putida* GPo1 is prone to biofilm formation, which can limit the stability and reproducibility of fermentation experiments and should be avoided.
Overall, cultivation under P-limitation could be a promising technique for fermentations with Gram-negative bacteria to reduce the product contamination by endotoxins.

3.5 Acknowledgments

We gratefully acknowledge the financial support by the Empa R&D fund. We also thank Ernst Pletscher and Dominik Noger for assisting with fermentations and for advices and Kathrin Grieder for ICP-OES measurements.
Chapter 4

Efficient recovery of low endotoxin medium-chain-length poly([R]-3-hydroxyalkanoate) from bacterial biomass

4.1 Introduction

New biomaterials, in particular degradable ones are needed for various medical applications (Hubbell 1995; Ueda and Tabata 2003). Today’s biomaterials of the third generation (Hench and Polak 2002) are designed to stimulate specific responses at the molecular level and to combine the concepts of bioactive and resorbable materials. Today’s polymeric and biodegradable systems used in medicine are mainly based on poly(lactic acid) (PLA), on poly(glycolic acid) (PGA), and on their co-polymers (Gomes and Reis 2004). Other biodegradable polymers have been proposed, but could not enter the market yet, due to lacking FDA approval (Gomes and Reis 2004).

One of the candidates is polyhydroxyalkanoate (PHA), a class of biodegradable and biocompatible polyesters with many potential applications in the medical field, such as heart valve scaffolds (Sodian et al. 2000a; Sodian et al. 2002), pulmonary conduits (Stock et al. 2000), sutures, screws, bone plates, repair patches, stents, bone marrow scaffolds and many others over the last years as recently reviewed by Chen and Wu (Chen and Wu 2005). In particular the scientific and industrial research on elastomeric PHA with medium-chain-length side-chains (mcl-PHA, containing 3-hydroxyalkanoate monomers from C_6-C_{14}) has been intensified in the last years (Chen et al. 2005; Sodian et al. 2002; Sodian et al. 2000b; Stock et al. 2000; Williams et al. 1999; Zinn et al. 2001).

Mcl-PHA is synthesized by fluorescent pseudomonads belonging to the rRNA homology group I (Huisman et al. 1989) and serves as an intracellular carbon and energy storage compound. Its biosynthesis can be triggered by appropriate growth conditions such as phosphorus and nitrogen limitation (Witholt and Kessler 1999). Mcl-PHA is stored in distinct granules that are coated by amphiphilic phospholipids and proteins (Pötter and Steinbüchel 2004).

Along with cell growth, all of the Gram-negative production strains produce lipopolysaccharides (LPS) as an integral part of the outer membrane (Petsch and Anspach 2000). LPS are pyrogenic and their concentration in a medical product is strictly regulated. The US-American Food and Drug Administration has given clear
regulations on the maximum endotoxin content of medical devices, namely 20 EU per medical device (U.S. Department of health and human services 1997).

There are currently two well-established approaches for recovering PHA: solvent extraction and aqueous digestion of non-PHA materials. The former one uses an organic solvent to extract PHA from dried biomass, whereas the latter one involves chemical agents or enzymes to lyse bacteria and thus liberating the PHA granules forming a latex (Choi and Lee 1999; de Koning et al. 1997; de Koning and Witholt 1997; Ling et al. 1997; Taniguchi et al. 2003; Yu and Chen 2006). To purify and depyrogenize PHA in latex form, oxidizing agents like hydrogen peroxide and sodium hypochlorite as well as sodium hydroxide were applied (Lee et al. 1999; Sevastianov et al. 2003; Williams et al. 1999). However, oxidizing agents may result in polymer degradation as was reported for the treatment with sodium hypochlorite (Taniguchi et al. 2003) and usually do require additional, expensive processes like ultracentrifugation and microfiltration (de Koning and Witholt 1997; Marchessault et al. 1995). In general, the PHA material obtained by this method is of low purity (Kessler et al. 2001) and therefore not suitable for medical applications.

Various organic solvents can be used to extract mcl-PHA from dried biomass (Chen et al. 2001; Kessler et al. 2001; Williams et al. 1999). The lyophilized biomass is commonly extracted with a chlorinated solvent and the resulting PHA solution is filtered (see Fig. 4.1A). In order to separate PHA from co-extracted impurities (e.g. lipids and proteins), the polymer solution is usually mixed with a non-solvent like methanol which induces precipitation of the polymer, leaving most contaminants in solution. The extraction conditions and further processing steps strongly affect the recovery and purity of the resulting PHA. Although polymer purities above 90% (w/w) are easily obtained by a solvent extraction followed by a non-solvent precipitation, the amount of endotoxins can still reach high levels (Sevastianov et al. 2003). Typically values of more than 100 EU g$^{-1}$ of polymer were found in a commercially available PHA, namely poly(3-hydroxybutyrate) (Williams et al. 1999).

In this study, we investigated an alternative method for the purification of mcl-PHA to obtain a polymer of high purity and low endotoxicity, namely by the heat driven extraction with a solvent and the selective precipitation induced by temperature reduction (see Fig. 4.1B). Special attention was paid to minimize the contamination with endotoxins.
Fig. 4.1: Schematic of PHA recovery: A) extraction and non-solvent precipitation, B) extraction and temperature-controlled precipitation.

4.2 Materials and Methods

Biosynthesis. Polyhydroxyoctanoate (PHO) consisting of 87% (w/w) 3-hydroxyoctanoate and 13% (w/w) 3-hydroxyhexanoate was produced by continuous cultivation of Pseudomonas putida GPO1 (Durner et al. 2000) in a 16 L bioreactor (Bioengineering, Wald, Switzerland). The dilution rate was set to 0.1 h⁻¹, the ammonium content of the minimal medium was 800 mg N L⁻¹ and the carbon feed rate was adjusted to obtain a carbon to nitrogen ratio of 12 g g⁻¹. The fermentation broth was then concentrated with a continuous centrifuge (Cepa Z61H, Lahr, Germany) at 17’500 x g and a flow rate of 50 L h⁻¹. The paste was lyophilized for 48 h, ground, and the dry biomass was stored in a closed container at -25 °C until extraction.

Analysis: Gel permeation chromatography (GPC). Molecular weights (number-average (Mn) and weight-average (Mw)) were determined by gel permeation chromatography (TDA 302, Viscotek, Waghausel-Kirrlach, Germany) equipped with a viscometer, RI- and light-scattering detector. The system was calibrated by using 2 polystyrene standards (Polycal, Viscotek) with known molecular weights and intrinsic viscosities (standard 1: Mw=115 kDa, Mn=112 kDa, IV=0.519, standard 2: Mw=250 kDa, Mn=100 kDa, IV=0.843). Forty mg of PHO-sample was dissolved in 10 mL of THF over night, filtered through a 1.0 µm nylon filter, and aliquots of 100 µL were
chromatographed with pure THF as solvent phase through three GPC columns (Mixed bed, Viscotek) at 35 °C and a flow rate of 1 mL min⁻¹.

**Gas chromatography (GC).** The purity of PHO and its composition with respect to alkanoate monomers were determined by gas chromatography. About 50 mg of polymer was transferred into a 20 mL Pyrex tube. Five mL of methylene chloride containing 10.0 mg mL⁻¹ of 2-ethyl-2-hydroxybutyric acid as internal standard and 5 mL of a 20/80 (v/v) mixture of HCl (37%) and n-propanol were added for the propanolysis at 80 °C for 16 h. Subsequently, the sample was cooled on ice and 8 mL of demineralized water was added. The mixture was vigorously shaken, the phases were separated, and the aqueous phase was removed. The organic phase was dried over Na₂SO₄, neutralized by adding Na₂CO₃, and filtered through a 1.0 µm nylon filter. One µL of this sample was injected into the GC (GC 8575 Mega 2, Fisons Instruments, Rodano, Italy) onto a Supelcowax-10: 30 m x 0.32 mm column with a film thickness of 0.5 µm (Supelco, Bellefonte, USA) at a split ratio 1:10 and an initial temperature 120 °C, which was raised at 10° min⁻¹ to 280 °C. Detection was performed by FID detection.

**Limulus amebocyte lysate (LAL)-test.** The endotoxicity was measured using a chromogenic LAL endpoint assay (QCL-1000, Cambrex, New Jersey, USA). About 300 mg of PHO were placed in a 15 mL pyrogen-free centrifuge tube (Corning, New York, USA) and heated up to 80 °C for 24 h to obtain a smooth polymer surface. Afterwards 6 mL of pyrogen-free water (Cambrex) was added. The tubes were placed in a rotary shaker and incubated for 24 h at 37 °C under constant shaking at 180 rpm to extract the endotoxins from the polymer. The aqueous endotoxin-solution was directly used for the LAL-test or diluted 1:10 or 1:100 if the concentration of endotoxins was high. Each analysis was carried out at least twice.

**Experimental setup.** For the recovery of PHO, we applied the solvent extraction method with precipitation by a non-solvent (standard extraction, Fig. 4.1A), and temperature-controlled precipitation, which does not require a non-solvent (Fig. 4.1B).

**Standard extraction.** Five gram of dry biomass containing about 30% (w/w) PHO were extracted with 75 mL of different organic solvents - methylene chloride (CH₂Cl₂), ethyl acetate, acetone, n-hexane, tetrahydrofuran (THF) and 2-propanol of purum grade or higher (Fluka, Buchs, Switzerland) - for 24 h at room temperature
under intense stirring. The biomass to solvent ratio of 1:15 (g mL\(^{-1}\)) had been identified as optimal in a preliminary test (data not shown) and was applied for all extractions. The suspension was filtered through a regenerated cellulose membrane filter (1.0 µm, Schleicher & Schuell Microscience GmbH, Dassel, Germany) with a pressure filtration unit (200 mL stainless steel, Sartorius AG, Goettingen, Germany) by applying an overpressure of typically 3 bars to obtain a clear PHO solution. The polymer solution was either evaporated or concentrated by rotary evaporation to 1/5 of the initial volume and then precipitated at -20 °C by adding 10 times the volume of methanol. Finally the polymer was dried under vacuum at 40 °C for 48 h. The resulting polymers had molecular weights of about 200 kDa (M\(_w\)) and 100 kDa (M\(_n\)) and hence a polydispersity of 2.0.

**Temperature dependent solubility.** About 1 g of pure PHO was dissolved in 100 mL organic solvent at 40 °C under intense stirring. The solution was cooled to 0 °C in an ice bath under gentle stirring. After 4 hours the solution was decanted. The precipitate was dried and the solvent was evaporated from the solution to determine the ratio between precipitated and dissolved PHO.

**Temperature-controlled extraction and precipitation.** Five gram of dried biomass was extracted with 75 mL of \(n\)-hexane at different temperatures under intense stirring for 24 h. The suspension was filtered at the same temperature as the extraction took place. In order to study the influence of temperature on the solubility of PHO in \(n\)-hexane, the polymer solution was kept at a temperature between +20 °C and -10 °C under gentle stirring for 24 h. The precipitated polymer was decanted and dried under vacuum at 40 °C for 48 h.

**Re-dissolution and precipitation.** For further purification, dried PHO (extracted with \(n\)-hexane at 50 °C and precipitated at 0 °C) was re-dissolved in 2-propanol (2% (w/v)) at 45 °C under intense stirring for 8 h. PHO was precipitated by cooling the solution to a temperature between 30 °C and 0 °C under gentle stirring for 24 h. Finally the polymer was dried under vacuum at 40 °C for 48 h.

**Calculation.** The purity and the recoveries of PHO were calculated according to the following equations:
\[ P_E = \sum \frac{m_{3-\text{OH}x}}{m_E} \cdot 100 \quad [% \text{w/w}] \quad [1] \]

\[ P_P = \sum \frac{m_{3-\text{OH}x}}{m_P} \cdot 100 \quad [% \text{w/w}] \quad [2] \]

\[ X_{\text{PHO}} = \sum \frac{m_{3-\text{OH}x}}{m_B} \cdot 100 \quad [% \text{w/w}] \quad [3] \]

where \( P_E \) is the purity of the extract, \( P_P \) the purity of the precipitate, \( X_{\text{PHO}} \) the PHO content in the dried biomass, \( m_E \) is the mass of the extract, \( m_P \) the mass of the precipitate, \( m_B \) the mass of the biomass, and \( m_{3-\text{OH}x} \) are the masses of the individual dehydrated 3-hydroxy acids (as present in the polymer) determined with GC as described above.

\[ R_E = \frac{m_E \cdot P_E}{m_B \cdot X_{\text{PHO}}} \cdot 100 \quad [% \text{w/w}] \quad [4] \]

\[ R_P = \frac{m_P \cdot P_P}{m_E \cdot P_E} \cdot 100 \quad [% \text{w/w}] \quad [5] \]

The recovery is either calculated for the extract relative to the total mass of PHO in the biomass (\( R_E \)), or for the precipitate relative to the mass of extracted PHO (\( R_P \)).

All measurements were carried out in duplicates. The bars in Fig. 4.2, Fig. 4.3 and Fig. 4.5 represent the uncertainty of measurement for the purity and the recovery (±5% and ±6%, respectively) and the maximum standard deviation for the endotoxicity (±10%). The uncertainty of measurement was calculated based on additional experiments not described in this paper.
4.3 Results and discussion

Standard extraction and precipitation. Biomass was extracted according to the conventional technique (Fig. 4.1A) to find solvents suitable to extract PHO selectively with a good recovery. As shown in Table 4.1, methylene chloride was the most efficient solvent at room temperature. 2-propanol and n-hexane were rather poor PHO extracting solvents. However, ethyl acetate, tetrahydrofurane, and acetone had recovery rates around 80% and provided PHO of purities > 80%. Especially ethyl acetate and acetone extracts contained less than 10% impurities. Furthermore n-hexane, methylene chloride, ethyl acetate, and acetone resulted in PHO of low endotoxicity.

Table 4.1: Recovery (RE), purity (PE or PP) and endotoxicity of PHO extracted from dried biomass with different solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solvent evaporated</th>
<th>Precipitated in MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_E$ [% w/w]</td>
<td>$P_E$ [% w/w]</td>
</tr>
<tr>
<td>n-hexane</td>
<td>53</td>
<td>93</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>86</td>
<td>78</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>80</td>
<td>92</td>
</tr>
<tr>
<td>THF</td>
<td>80</td>
<td>84</td>
</tr>
<tr>
<td>Acetone</td>
<td>83</td>
<td>92</td>
</tr>
<tr>
<td>2-propanol</td>
<td>23</td>
<td>66</td>
</tr>
</tbody>
</table>

The subsequent precipitation of PHO with methanol drastically increased the purity of the polymer. Purities close to 100% could be reached (Table 4.1). At the same time, only small amounts of PHO, usually less than 5%, were lost.
Temperature-controlled precipitation. To identify solvents capable of dissolving PHO in a temperature dependent manner (Fig. 4.1B), PHO solutions of 1% (w/v) in organic solvent were prepared. The concentration of 1% (w/v) was chosen because it is typical for the concentration reached after biomass extraction with an organic solvent at a ratio 1:15 (w/v). The polymer solutions were cooled down to 0 °C and sol- and gel-phase were separated by decantation.

There were only two solvents with sufficiently different solubilities at 40 °C and 0 °C: 2-propanol and n-hexane (Table 4.2). Nearly all dissolved polymer could be recovered from 2-propanol whereas in n-hexane about 26% of the polymer remained in solution. The other solvents showed no polymer precipitation when the temperature was adjusted to 0 °C, consequently they are not suitable for a temperature induced precipitation of PHO. Eventually polymer precipitation would have taken place at significantly lower temperatures but lower purities had to be expected due to co-precipitation of impurities present in the solution.

Of the two solvents with a suitable temperature-dependent behavior, 2-propanol is much less suitable for extracting PHO since it co-extracted a lot of cellular compounds and thus resulted in PHA of low purity (Table 4.1). In contrast, n-hexane selectively extracted PHO from biomass and was therefore suitable for a combined extraction / temperature-controlled precipitation.

Table 4.2: Fractions of precipitated PHO after cooling the polymer solutions to 0 °C.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Precipitated polymer [% w/w]</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane</td>
<td>74</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0</td>
</tr>
<tr>
<td>THF</td>
<td>0</td>
</tr>
<tr>
<td>Acetone</td>
<td>0</td>
</tr>
<tr>
<td>2-propanol</td>
<td>97</td>
</tr>
</tbody>
</table>
Temperature optimization of PHO extraction. After having identified \( n \)-hexane as the solvent with the most promising properties, we optimized the extraction step with respect to temperature. PHO was extracted with \( n \)-hexane at temperatures between 20 °C and \( n \)-hexane’s boiling point at 69 °C. The recovery increased considerably with rising extraction temperature. A plateau was reached at 50 °C (Fig. 4.20). The purity of the resulting PHO slightly decreased with increasing temperature, but still remained close to 90% at the boiling point. In contrast, the endotoxicity increased dramatically with the extraction temperature from 10 to >1'000 EU g\(^{-1}\) polymer (Fig. 4.2). Hence the co-extraction of LPS from biomass is much more pronounced at higher extraction temperatures. The molecular weight of PHO increased moderately with increasing temperature, indicating that high molecular weight PHO dissolved better above 20 °C than below (Table 4.3). Consequently, the extraction with \( n \)-hexane was very efficient above 50 °C, but due to the increased contamination with endotoxins, an extraction temperature below 50 °C was more reasonable.

In subsequent experiments, dry biomass was extracted with \( n \)-hexane at 50 °C for 24 h. This temperature was chosen in order to clearly document a potential endotoxin reduction effect of the precipitation.

Fig. 4.2: Recovery, purity and endotoxicity for PHO extracted with \( n \)-hexane at different temperatures.
Table 4.3: Weight averaged molecular weight ($M_w$), number averaged molecular weight ($M_n$) and polydispersity (D) of PHO extracted from biomass with n-hexane at different temperatures.

<table>
<thead>
<tr>
<th>Extraction temperature [°C]</th>
<th>$M_w$ [kDa]</th>
<th>$M_n$ [kDa]</th>
<th>D [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>190</td>
<td>96</td>
<td>1.97</td>
</tr>
<tr>
<td>30</td>
<td>202</td>
<td>104</td>
<td>1.95</td>
</tr>
<tr>
<td>40</td>
<td>193</td>
<td>98</td>
<td>1.97</td>
</tr>
<tr>
<td>50</td>
<td>209</td>
<td>113</td>
<td>1.86</td>
</tr>
<tr>
<td>60</td>
<td>221</td>
<td>114</td>
<td>1.95</td>
</tr>
<tr>
<td>69</td>
<td>212</td>
<td>111</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Temperature optimization of PHO precipitation. To investigate the optimum precipitation temperature of PHO from n-hexane, the polymer solution was cooled after filtration to temperatures between +20 and -10 °C. First precipitations were observed at 20 °C. The amount of precipitated polymer increased further with decreasing temperature and may have reached a maximum between 5 and 0 °C. Surprisingly, precipitation became less efficient at a temperature below 0 °C as shown in Fig. 4.3. Apparently, PHO formed a dispersion with n-hexane at temperatures below 0 °C and precipitated very slowly. About 80% of the extracted PHO could be precipitated at best. However, an improved recovery could be obtained when the polymer solution was concentrated previously, e.g. by solvent evaporation.

The purity of the resulting polymer was close to 100% for precipitation temperatures down to 5 °C, and declined for lower temperatures (Fig. 4.3). This indicates that non-polar contaminants co-precipitated significantly below 0 °C. The endotoxicity increased at low temperatures (Fig. 4.3) in parallel with the observed precipitation of other impurities.
Fig. 4.3: Recovery, purity and endotoxicity for PHO extracted with n-hexane at 50 °C, precipitated at different temperatures (20 °C: endotoxicity not determined).

Gel permeation chromatography (GPC) showed that particularly the high molecular weight fractions of the polymer precipitated at higher temperatures and that the low molecular weight fractions remained in solution as shown in Fig. 4.4. This led to a decrease of the polydispersity ($M_w/M_n$) from 1.86 to 1.39 at 10 °C (Table 4.4). This is important since decreasing the amount of low molecular weight species prevents a rapid and extensive release of mono- or oligomers in medical (implants) and pharmaceutical (drug carriers) applications. For some polymers in medical applications it has been reported that the release of low molecular weight components led to a drastic decrease of the pH near the implant surface (Agrawal and Athanasiou 1997) or often caused inflammations (Eckelt et al. 2004).

Since the goal was to produce low endotoxin containing PHA at a high yield, it was concluded that the optimal precipitation temperature was 0 °C taking into account that the polydispersity was somewhat larger ($D = 1.56$).
Fig. 4.4: Normalized molecular weight distribution of PHO extracted with \( n \)-hexane at 50 °C, precipitated at different temperatures.

Table 4.4: Weight averaged molecular weight (\( M_w \)), number averaged molecular weight (\( M_n \)) and polydispersity (D) of PHO extracted with \( n \)-hexane at 50 °C, precipitated at different temperatures (starting material: \( M_w=209 \text{ kDa}, M_n=113 \text{ kDa}, D=1.86 \)).

<table>
<thead>
<tr>
<th>Precipitation temperature* [°C]</th>
<th>( M_w ) [kDa]</th>
<th>( M_n ) [kDa]</th>
<th>D [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>363</td>
<td>262</td>
<td>1.39</td>
</tr>
<tr>
<td>5</td>
<td>233</td>
<td>157</td>
<td>1.48</td>
</tr>
<tr>
<td>0</td>
<td>228</td>
<td>146</td>
<td>1.56</td>
</tr>
<tr>
<td>-5</td>
<td>204</td>
<td>121</td>
<td>1.70</td>
</tr>
<tr>
<td>-10</td>
<td>217</td>
<td>125</td>
<td>1.73</td>
</tr>
</tbody>
</table>

*Molecular weight analysis could not be carried out for the sample at 20 °C because of insufficient polymer recovery.

2-propanol re-dissolution and precipitation. In order to further improve the purity of PHO above 97%, we investigated the re-dissolution of the purified polymer in 2-propanol followed by another temperature-controlled precipitation, because 2-propanol was very efficient in co-extracting cellular impurities (see above). Thus we
proposed that the sequential usage of \( n \)-hexane and 2-propanol for temperature-controlled precipitation has a beneficial effect on the purity of PHA since impurities may have a different affinity to these solvents. PHO obtained from \( n \)-hexane extraction at 50 °C and precipitated at 0 °C was re-dissolved in 2-propanol at 45 °C to a concentration of 20 g L\(^{-1}\). The solution was then cooled to temperatures between 30 and 0 °C. The PHO recovery increased with decreasing temperatures as shown in Fig. 4.5. At 25 °C already 80% of the polymer had precipitated. At 0 °C nearly 100% of the PHO was recovered. The purity was close to 100% for all temperatures. The endotoxicity was lowered to less than 10 EU g\(^{-1}\) polymer at all temperatures, except at 0 °C (Fig. 4.5). A temperature of 10 °C resulted in the lowest endotoxicity of PHO with 2 EU g\(^{-1}\) polymer. GPC measurements again showed a fractionation effect according to the molecular weight (Fig. 4.6 and Table 4.5). High molecular weight fractions precipitated at elevated temperatures, whereby the low molecular weight fractions remained in solution. The polydispersity was decreased from 1.50 to a minimum of 1.26.

![Graph of Recovery, Purity, and Endotoxicity](image-url)

**Fig. 4.5:** Recovery, purity and endotoxicity for PHO re-dissolved in 2-propanol at 40 °C and precipitated at different temperatures.
Chapter 4

Fig. 4.6: Normalized molecular weight distribution of PHO re-dissolved in 2-propanol and precipitated at different temperatures.

Table 4.5: Weight average molecular weight ($M_w$), number average molecular weight ($M_n$) and polydispersity (D) of PHO re-dissolved in 2-propanol and precipitated at different temperatures (starting material: $M_w$=228 kDa, $M_n$=146 kDa, D=1.56).

<table>
<thead>
<tr>
<th>Precipitation temperature [°C]</th>
<th>$M_w$ [kDa]</th>
<th>$M_n$ [kDa]</th>
<th>D [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>321</td>
<td>255</td>
<td>1.26</td>
</tr>
<tr>
<td>25</td>
<td>260</td>
<td>199</td>
<td>1.31</td>
</tr>
<tr>
<td>20</td>
<td>244</td>
<td>184</td>
<td>1.33</td>
</tr>
<tr>
<td>15</td>
<td>234</td>
<td>159</td>
<td>1.47</td>
</tr>
<tr>
<td>10</td>
<td>231</td>
<td>156</td>
<td>1.48</td>
</tr>
<tr>
<td>5</td>
<td>239</td>
<td>159</td>
<td>1.50</td>
</tr>
<tr>
<td>0</td>
<td>227</td>
<td>156</td>
<td>1.46</td>
</tr>
</tbody>
</table>
4.4 Conclusions

Temperature-controlled extraction and precipitation can be effectively used to increase the purity and reduce the endotoxin content of microbial PHO samples. For this reason, it represents a valuable alternative to the classical approach where the PHA-solution is mixed with an excess of non-solvent for precipitating mcl-PHA. Our method yields in a polymer of high quality, is very simple and omits the disadvantages of a non-solvent precipitation (e.g. separation and recycling of solvents and non-solvents).

\( \text{N-hexane} \) was useful for a quantitative extraction of PHO at a temperature around 50 °C. By decreasing the extraction temperature to 40 °C or below, the recovery decreased moderately but the PHA contained much less endotoxins. Precipitation induced by cooling led to PHO of high purity and low endotoxicity, leaving most impurities in solution. However, a certain loss of polymer can not be prevented except through a pre-concentration of the polymer solution. Especially the low molecular weight fractions of PHO hardly precipitated.

The temperature dependent capacity to dissolve PHO made 2–propanol a promising candidate for PHO extraction from biomass, but it dissolved other cell components as well, which made it less suitable for the first extraction step. However, its increased polarity relative to \( n \)-hexane led us to reason that it might be an excellent solvent to re-dissolve PHO and selectively remove endotoxins. Indeed, endotoxin concentrations of lower than 10 EU g\(^{-1}\) of polymer could be reached. Additionally, the polymer polydispersity and the molecular weight can be controlled by a temperature-controlled precipitation in order to achieve a more homogenous degradation.

The solubility of various mcl-PHA in organic solvents can differ notably and therefore the presented method might not be applicable to all mcl-PHAs without adjustments. Recently, the method could be applied to a co-polymer produced from octanoic and 10-undecenoic acid, where the relative composition was 50:50 w% (Hartmann et al. 2006).

Concluding, we present a simple and very efficient method to produce PHO that complies with the endotoxin requirements of the FDA for biomedical applications.
such as implants and drug delivery systems (U.S. Department of health and human services 1997).

4.5 Acknowledgement

We gratefully acknowledge the financial support by Empa. We also thank Ernst Pletscher and René Hartmann for their help with fermentations, Thomas Ramsauer for assisting with polymer extractions and Manfred Schmid and Elisabeth Michel for supporting GPC and GC analyses.
Chapter 5

Biocompatibility of untreated and chemically modified medium-chain-length poly(3-hydroxyalkanoates)

The results of this chapter were obtained in collaboration with:

Katharina Maniura and Philipp Spohn – cytotoxicity experiments
Sarah Cartmell – cell culturing on PHA coatings
Sarah Rathbone – cell culturing on PHA coatings

Patrick Furrer has carried out the following parts: PHA biosynthesis, PHA extraction and purification, chemical derivatisation of PHA, solvent casting and interpretation of cell culturing experiments.
5.1 Introduction

During the last two decades, significant advances have been made in the development of biocompatible and biodegradable materials for biomedical applications. The development of new biocompatible materials includes considerations that go beyond the absence of toxic effects: the stimulation of specific responses of tissue cells at the molecular level is the aim of so-called third generation biomaterials (Hench and Polak 2002).

The chemical composition and surface structure of biomaterials are mainly responsible for the interaction with biological systems. In the presence of serum, it is thought that attachment of cells is mediated through adsorbed proteins and the underlying surface chemistry controls protein adsorption. Through a protein layer, cell adhesion and spreading can be prevented or promoted (Mrksich 2000). Physical and chemical techniques can be used to modify the surface properties and hence to control protein adsorption and cell adhesion. For instance plasma treatment (Qu et al. 2005; Tezcaner et al. 2003), UV radiation (Shangguan et al. 2006; Viktor N. Vasilets et al. 2004), collagen immobilisation (Tesema et al. 2004) and chemical derivatisation with hydroxide (Rouxhet et al. 1998) have been used. Consequently, the wettability or hydrophilicity of surfaces, as indicated by the water contact angle, is of paramount importance for their interaction with cells. For osteoblasts, good proliferation was obtained for fibronectin-coated surfaces with a water contact angle of 50-90 ° (Kennedy et al. 2006).

Poly(3-hydroxyalkanoate) (PHA), a class of biodegradable polyesters, has the potential to become an important biomaterial for medical applications (Williams et al. 1999; Zinn et al. 2001). Due to lower crystallinity and increased elasticity, mcl-PHA offer physical properties that were quite different from scl-PHAs and give new possibilities for medical applications such as scaffolds for tissue engineering and as carrier for drug delivery. Functional groups in the side-chains of mcl-PHA allow to regulate its properties. In addition, chemical modification of functionalized PHAs enables further tailoring of the chemo-physical properties to suit particular applications (Hazer and Steinbuechel 2007). For instance, the side-chain alkenes of mcl-PHA were quantitatively transformed into hydroxyl groups with borane
tetrahydrofurane (Renard et al. 2005). The hydrophilicity of the polymer increased and the modified PHA was soluble in polar solvents like ethanol.

In vitro and in vivo studies have so far focused on short-chain-length PHA (scl-PHA) with monomers of 3-5 carbon atoms and rather few studies were carried out with medium-chain-length PHA (mcl-PHA) with monomers of 6-14 carbon atoms (Chen and Wu 2005; Sodian et al. 2000a; Sodian et al. 2000b; Sodian et al. 2000c).

The combination of polyhedral oligomeric silsesquioxane (POSS) molecules and polymers holds great promises for the future of biomedical devices, especially for cardiovascular interfaces (Kannan et al. 2005). It has been shown that POSS nanocomposites, unlike certain carbon nanotubes (Cui et al. 2005) are cytocompatible and hence suitable for tissue engineering (Kannan et al. 2005; Punshon et al. 2005). Further, It has been postulated that pendant nanocages containing silicon atoms form foci with increased surface free energy thus promoting the formation of an endothelial cell layer (endothelialization) and repelling coagulant proteins (Kannan et al. 2005; Silver et al. 1999). The combination of POSS to mcl-PHA would be of particular interest because the cell adhesion and proliferation on a mcl-PHA matrix could be drastically enhanced.

In this work different mcl-PHAs with saturated and unsaturated side-chains were produced and purified until the contamination with biological residues, in particular with endotoxins was very low. The biocompatibility of these PHAs was studied. Further, chemical modification was applied to hydrophobic mcl-PHAs with unsaturated side-chains to increase their hydrophilicity and to improve cell adhesion and proliferation. This was done by introducing carbonyl and carboxyl groups via reaction with ozone or by covalently binding POSS to the unsaturated side-chains.
5.2 Materials and Methods

Biosynthesis. Mcl-PHAs were produced by continuous cultivation of *Pseudomonas putida* GPo1 (ATCC 29347) as previously described (Ruth et al. 2007). Undecanoic, 10-undecenoic acid or a mixture of octanoic and 10-undecenoic acid were used as carbon source for the production of poly(3-hydroxyundecanoate) (PHUA), poly(3-hydroxy-10-undecenoate) (PHUE) and poly(3-hydroxyoctanoate-co-3-hydroxy-10-undecenoate) (PHOUE). A 16 L laboratory bioreactor (Bioengineering, Wald, Switzerland) with a working volume of 10 L was used. The dilution rate was set to 0.1 h\(^{-1}\), the ammonium content of the minimal medium was 800 mg N L\(^{-1}\) and the carbon feed rate was adjusted to obtain a carbon to nitrogen ratio of 12 g g\(^{-1}\). The fermentation broth was then concentrated with a continuous centrifuge (Cepa Z61H, Lahr, Germany) at 17’500 x \(g\) and a flow rate of 50 L h\(^{-1}\). The paste was lyophilized for 48 h, ground, and the dry biomass was stored in a closed container at -25 °C until PHA extraction.

PHA extraction. PHUA, PHUE and PHOUE were extracted from dried biomass with methylene chloride under stirring at room temperature for 24 h. After filtration, the polymer solution was concentrated to one fifth of its original volume, cooled to 0 °C and a tenfold excess of ethanol was added to induce precipitation. The polymer was separated from the liquid phase by decantation and dried under vacuum. For purification, the polymers were re-dissolved in methylene chloride, cooled to 0 °C and a 10 fold excess of ethanol was added for precipitation. The solvent was decanted and the PHA dried under vacuum.

Chemical derivatisation. PHOUE was linked with polyhedral oligomeric silsesquioxane (POSS) via a free radical addition reaction as described elsewhere (Hany et al. 2005). 1.9 g PHOUE, 14.0 g mercaptopropyl-isobutyl-POSS (POSS-SH) and 0.27 g azobisisobutyronitrile (AIBN) were dissolved in 100 mL toluene and heated to 75 °C for 18 h under nitrogen atmosphere. The solution was cooled to 0 °C and 1 L of methanol was added for precipitation. To remove unreacted POSS-SH, the polymer was dissolved in 200 mL methylene chloride and 1.5 L methanol was added at room temperature for the precipitation of PHOUE-POSS. This procedure was repeated four times for a complete removal of POSS-SH. The precipitate was dried under vacuum.
PHUE coatings in Petri dishes were treated with ozone generated with an ozoniser (Certizon 200, Sander, Uetze-Eltze, Germany) supplied with pure oxygen. The treatment time was set to 15 min for complete derivatisation of the double bonds on the polymer surface based on contact angle measurements.

**Solvent casting.** Small (d=4 cm) and large (d=19.5 cm) glass Petri dishes were coated with PHA for biocompatibility experiments after they had been depyrogenised by heating to 200 °C for 6 h. To this end, the polymer was dissolved in dichloromethane at a concentration of 2.5% w/v. The solution was filtrated (Nylon filter, 0.45 µm) and cast into depyrogenised Petri dishes. The polymer solution was slowly allowed to evaporate for 3 days at room temperature under an atmosphere nearly saturated with dichloromethane to avoid the formation of bubbles. After that, the polymer coatings were further dried under vacuum for 18 h at 40 °C. The resulting polymer coating had a thickness of about 100 µm. Petri dishes with PHA coatings were sterilised by x-ray previous to cell culturing.

**PHA coating purification.** For the cell culturing on PHA coatings, PHOUE-POSS and PHUE treated with ozone (PHUE-O3) were purified to remove impurities of the chemical derivatisation from the surface: PHOUE-POSS coatings were extracted with 3 mL of pure water for 3 h and with 3 mL of ethanol for 3 h and dried under vacuum. PHUE-O3 was only dried under vacuum for 18 h to remove volatile impurities.

**Endotoxin measurement.** The endotoxicity was measured using a chromogenic limulus amebocyte lysate (LAL) endpoint assay (QCL-1000, Cambrex, New Jersey, USA). About 100 mg PHA was dissolved in 3 mL methylene chloride and solvent cast into a depyrogenised glass Petri dish (d=4 cm). The solvent was slowly evaporated under a nearly saturated atmosphere as described above the polymer coatings were further dried under vacuum at 40 °C for 18 h. Three mL of pyrogen-free water (Cambrex) was added to the Petri dish and incubated at 37 °C for 24 h to extract the endotoxins from the polymer. The aqueous endotoxin-solution was directly used for the LAL-test. Each analysis was carried out at least twice.

**Water contact angle.** The water contact angle was determined using a Krüss G10 contact angle measuring system (Hamburg, Germany) and pure water (Chromasolv, Sigma-Aldrich). Three slides were coated for each polymer and two measurements with droplets of 30 µL were made on each slide.
**Gel permeation chromatography (GPC).** Molecular weights (number-average $M_n$ and weight-average $M_w$) were determined by gel permeation chromatography (TDA 302, Viscotek, Waghausel-Kirrlach, Germany) equipped with a viscometer, RI- and light-scattering detector. The system was calibrated by using 2 polystyrene standards (Polycal, Viscotek) with known molecular weights and known intrinsic viscosities (standard 1: $M_w = 115$ kDa, $M_n = 112$ kDa, $IV=0.519$, standard 2: $M_w = 250$ kDa, $M_n = 100$ kDa, $IV=0.843$). Then, 40 mg of PHA was dissolved in 10 mL of THF over night, filtered through a 1 µm nylon filter, and aliquots of 100 µL were chromatographed with pure THF as solvent phase through three GPC columns (Mixed bed, Viscotek) at 35 °C and a flow rate of 1 mL min$^{-1}$.

**Gas chromatography.** Quantitative analysis of PHA has been performed by methanolysis catalyzed by BF$_3$ as described in chapter 2.

**Cytotoxicity according to ISO10993-5**

To test if a material releases biologically active substances to an aqueous environment, the material was incubated for 3 days in water. Fibroblasts of type 3T3 were cultured with these extracts on 96 well plates. After 5 days the cell cultures were analysed to determine the influence of the extract.

**Extraction of water soluble PHA contaminations.** The polymer coatings prepared by solvent casting in Petri dishes (d=19.5 cm) had a thickness of about 10 µm and a surface of 300 cm$^2$. An aliquot of 25 mL of water containing 1% penicillin-streptomycin-neomycin (PSN) antibiotics (Invitrogen, Basel, Switzerland) was added to the Petri dish to extract substances from the PHA coating and incubated for 72 h at 37 °C. The pH was initially adjusted to 7.4 with NaHCO$_3$. The aqueous extract was diluted with water and supplemented with 800 µL of Dulbecco’s minimal essential medium (DMEM, Invitrogen), 300 µL NaHCO$_3$ 7.5%, 400 µL of foetal bovine serum (FBS, Invitrogen) and 80 µL of PSN antibiotics to obtain solutions containing 0, 10, 20, 50 and 70 % of the aqueous extract.

**Cell culturing.** Each well was incubated with 200 µL of cell suspension containing about 7500 3T3 cells per mL for 24 h. The medium was gently removed and 200 µL of the diluted extract was added. After 5 days of incubation at 37 °C and a gas atmosphere containing 5% CO$_2$, DNA from permeabilised cells was measured with
the Hoechst 33258 DNA stain (Invitrogen) and total protein was determined with the bicinchonin acid protein assay kit (Pierce, Nr. 23225, Rockford Illinois) according to the manufacturers protocols. To measure the metabolic activity, the mitochondrial activity was determined by the MTT assay (Mosmann 1983) and the activity of lysosomes was measured with the neutral red assay (Borenfreund et al. 1985).

**Cell culturing on PHA coatings**

**Cell culture.** Mouse connective tissue fibroblasts were bought (cell line L929 – ECACC, 85011425, lot 04H008, Sigma, Poole, UK) and cultured in 88% DMEM high glucose (Biosera L0106, East Sussex, UK), supplemented with 10% FCS (504002, batch S1900-Biosera), 1% antibiotics (streptomycin and penicillin L00100, Biosera), 1% L-glutaraldehyde (G7513, Sigma-Aldrich), and incubated at 37 °C with a gas atmosphere containing 5% CO₂ in T175 culture flasks (Starstedt T175 vented red cap 83.1813.002, Leicester, UK). The cells were passaged 2 times and seeded at a density of 100’000 cells/well into the small PHA-coated Petri dishes and into the wells of Costar polystyrene (PS) 6-well plates (Corning, No. 3516, Massachusetts, USA) and incubated to the required time point (7 days and 14 days). The medium was changed every 4 days throughout the incubation period.

The cell viability was measured with the live/dead assay kit (Invitrogen, L3224). To quantitate double-stranded DNA in solution, the PicoGreen® assay was used (Invitrogen, P7589). Cells adhered to PHA and PS and cells in suspension were measured separately. The manufacturers’ protocols were used for these two assays. The hydroxyproline content was considered as an indicator for the collagen production. It was determined as described elsewhere (Kivirikko et al. 1967). Probability values of less than 0.05 (P<0.05) were considered to be significantly different (2-tailed student T-test, n=5).
5.3 Results and discussion

5.3.1 Physical and chemical properties of PHAs

The purity of all PHAs was >95 w% according to GC measurements (Table 5.1). The endotoxic activity of the polymer coatings was decreased below the detection limit and was below 1 endotoxic unit per gram (EU g\(^{-1}\)). The molecular weights were similar for PHUA, PHUE, PHOUE and PHUE-O3 (M\(_w\) ≈ 200 kDa) and only for PHOUE-POSS clearly increased, which was due to the linked POSS groups. The water contact angle of untreated PHAs (PHUA, PHUE and PHOUE) was between 104\(^\circ\) and 109\(^\circ\), whereas the POSS-derivatised PHOUE and the ozone-treated PHUE showed lower values of 95\(^\circ\) and 73\(^\circ\) respectively. The monomeric composition of all PHAs prior to chemical derivatisation is given in Table 5.2.

Table 5.1: Extraction, purification and derivatisation techniques applied for the production of different mcl-PHAs and their physical properties.

<table>
<thead>
<tr>
<th>PHAs</th>
<th>Extraction</th>
<th>Purification</th>
<th>Derivatisation</th>
<th>Contact angle</th>
<th>M(_w)</th>
<th>M(_n)</th>
<th>Purity</th>
<th>Endotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>solvent-</td>
<td>solvent-</td>
<td></td>
<td>[(^\circ)]</td>
<td>[kDa]</td>
<td>[kDa]</td>
<td>[w%]</td>
<td>[EU g(^{-1})]</td>
</tr>
<tr>
<td></td>
<td>non solvent</td>
<td>non solvent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHUA</td>
<td>CH(_2)Cl(_2)-EtOH</td>
<td>CH(_2)Cl(_2)-EtOH 2x</td>
<td>-</td>
<td>109</td>
<td>168</td>
<td>91</td>
<td>&gt;95</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PHUE</td>
<td>CH(_2)Cl(_2)-EtOH</td>
<td>CH(_2)Cl(_2)-EtOH 2x</td>
<td>-</td>
<td>104</td>
<td>231</td>
<td>115</td>
<td>&gt;95</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PHOUE</td>
<td>CH(_2)Cl(_2)-EtOH</td>
<td>CH(_2)Cl(_2)-EtOH 2x</td>
<td>-</td>
<td>108</td>
<td>255</td>
<td>151</td>
<td>&gt;95</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PHOUE-POSS</td>
<td>CH(_2)Cl(_2)-EtOH</td>
<td>CH(_2)Cl(_2)-EtOH 2x, POSS-SH</td>
<td>CH(_2)Cl(_2)-EtOH 4x</td>
<td>95</td>
<td>910</td>
<td>420</td>
<td>&gt;95</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PHUE-O3</td>
<td>CH(_2)Cl(_2)-EtOH</td>
<td>CH(_2)Cl(_2)-EtOH 2x, Ozone</td>
<td>CH(_2)Cl(_2)-Ozone</td>
<td>73</td>
<td>231</td>
<td>115</td>
<td>&gt;95</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

\(^2\) Purification after chemical derivatisation
Table 5.2: Monomeric composition of mcl-PHAs determined by GC after transesterification.

<table>
<thead>
<tr>
<th>PHAs</th>
<th>Monomeric composition [mole%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C5:0</td>
</tr>
<tr>
<td>PHUA</td>
<td>2.8</td>
</tr>
<tr>
<td>PHUE</td>
<td></td>
</tr>
<tr>
<td>PHOUE</td>
<td>5.1</td>
</tr>
<tr>
<td>PHOUE-POSS</td>
<td>5.1</td>
</tr>
<tr>
<td>PHUE-O3</td>
<td></td>
</tr>
</tbody>
</table>

a) The notation Cn:x was used to denote the 3-hydroxy acids, where n is the number of carbon atoms and x indicates the position of the double bond (0: no double bond present).

5.3.2 Cytotoxicity according to the eluate test

A critical point for the application of PHA (and other polymers) in the medical field is the effect of water soluble substances contained in the polymer. To proof that no toxic substances can diffuse from the polymer matrix to an aqueous environment the cytotoxicity test according to ISO 10993-5 is commonly applied. Accordingly, PHA coatings were extracted with water for 3 days and 3T3 fibroblasts were incubated with diluted extracts for 5 days and finally the proliferation was determined. For pure PHAs without chemical derivatisation a toxic effect in the aqueous eluate could hardly be seen (Fig. 5.1A - C) in contrast to chemically derivatised PHAs where cell proliferation was strongly reduced (Fig. 5.1D - E).
Fig. 5.1: Cytotoxic activity of aqueous extracts from PHUA (A), PHUE (B), PHOUE (C), PHOUE-POSS (D) and PHUE-O3 (E). The toxic activity was determined with 3T3-fibroblasts by • DNA, ○ MTT, △ protein and ▼ neutral red assays.
For PHUA, and PHUE no toxic effect could be seen with the DNA, MTT, protein and neutral red assay (Fig. 5.1A - B). Their values varied between 90 and 115% relative to the control, but there was no increase in the effect with increasing concentration. Hence, these polymers appear to be free of biologically active impurities that could be solubilised in an aqueous environment. For PHOUE a cytotoxic effect was measured at the highest extract concentration where the values for DNA, MTT and protein were reduced to about 80% of the control (Fig. 5.1C). Apparently, PHOUE contained small amounts of biologically active impurities.

For PHOUE-POSS the cell proliferation and activity was decreased after 5 days to about 60% (Fig. 5.1D). This toxic effect was attributed to side-products formed during chemical derivatisation. Although the derivatised PHA was thoroughly purified by dissolution-precipitation until no impurities of low molecular weight could be detected by GPC, high toxicity was still measured.

Even more drastically the relative values of DNA, MTT, protein and neutral red decreased for PHUE-O3. They reached around 20% at the highest extract concentration (Fig. 5.1E). It has been reported that formaldehyde and small amounts of formic acid are generated during oxidation of alkenes with ozone (Dubowski et al. 2004; Eliason et al. 2003). Adsorption of these products on the polymer surface, or trapping in the polymer combined with slow diffusion can explain the toxic effect we measured. Further purification of PHOUE-POSS and PHUE-O3 was required after chemical derivatisation and was applied before cell culturing on PHA coatings.

### 5.3.3 Cell culturing on PHA coatings

Mouse connective tissue fibroblasts were seeded directly on the PHA coatings to analyse their interaction with the polymer surface. Polystyrene (optimised for cell attachment) was used as control material.

**Unmodified PHAs: PHUA, PHUE and PHOUE**

Proliferation had occurred during the 7 days according to the Picogreen assay. About 90%, 230% and 60% more cells were found on the PHUA, PHUE and PHOUE coating, respectively in comparison to the control (Fig. 5.2). Most cells were attached to the mcl-PHA surface and only few cells (~ 2%) were suspended in the medium.
Consequently, the loss of cells during medium change was negligible. This observation was similar for all mcl-PHA coatings.

![Cell number per well](image)

**Fig. 5.2:** Cell numbers of adhered cells on PHUA, PHUE and PHOUE coatings and corresponding controls (control PHUA, control PHUE and control PHOUE) after 7 days, determined with the Picogreen assay.

The results of the live/dead assay for PHUA and PHUE showed that very few viable cells were present on these PHA coatings in comparison to the control (Fig. 5.3a-c). This is in contrast to the measurements made with the Picogreen assay. The cell film on the PHA coating was very confluent before the staining procedure. However, before the live/dead staining was carried out, the cell density was not evenly distributed on the PHUE coating but found in patches. The surface properties of the PHA coating may have prevented the cells from adhering strongly, where they became dislodged and washed away during washing steps of the live/dead assay. Still, cell adhesion was strong enough to prevent a significant loss of cells during routine medium change as shown with the Picogreen assay.

Contrarily, the live/dead assay for PHOUE showed that this coating had a high density of viable cells, comparable to the cell density of the control (Fig. 5.3d and e). This indicates that the surface properties were suitable for providing strong cell attachment.
The results of the hydroxyproline assay, which is used as an indicator for the collagen production, showed that there was a higher concentration of hydroxyproline in the PS control than in the coatings of PHUA, PHUE and PHOUE as exemplified for PHUA in Fig. 5.4. The hydroxyproline concentration was reduced to 35% for PHUA and PHUE and to 50% for PHOUE compared to the reference. However, for the PHUE coating there was a considerable error in the hydroxyproline concentration. This assay specifically measures the amount of hydroxyproline produced by
functional cells in the form of collagen. Hydroxyproline makes up for up to 13% of collagen. However, the cells on PHA coatings seemed to be significantly less functional than the control cells. Possibly, degradation products of PHA had effected gene expression of certain extracellular matrix genes, such as switching off the gene that produces hydroxyproline.

The results of these assays suggest that the PHUA, PHUE and PHOUE coatings had no toxic effect and were supporting some cell growth with limited collagen production. The surface properties of PHUA and PHUE may not suitable to allow strong cell adherence. Only for PHOUE good cell attachment was observed. The reason for improved cell adhesion was not clear. It could not be attributed to the unsaturated side-chains because the cell adhesion was weak for PHUE, which contained more unsaturated side-chains. Possibly the surface of PHOUE had an increased roughness, thereby easing cell adhesion.

![Graph showing hydroxyproline concentration](image)

**Fig. 5.4:** Hydroxyproline assay for cells on PHUA and PHOUE-POSS coatings and corresponding PS controls (control PHUA and control PHOUE-POSS) after 14 days. P = 9.45 x 10^{-10} and 0.029, respectively.

**Chemically modified mcl-PHAs: PHOUE-POSS and PHOUE-O3**

Similarly to PHOUE, a high cell number was observed with the Picogreen assay for the PHOUE-POSS coating. In contrast to this, the cell number on the PHOUE-O3 coating was nearly identical to the one of the control and hence smaller compared to all other PHA coatings.
For PHOUE-POSS many viable cells were observed with the live/dead assay (Fig. 5.5a). The density was comparable to the control. Hence, the cell adhesion was strong, similar to that of PHOUE. In analogy, the live/dead assay showed a high cell density for PHUE-O3 (Fig. 5.5c). Consequently, strong cell adhesion was obtained by treating PHUE with ozone.

![Fluorescence microscope images of live/dead-stained mouse connective tissue fibroblasts on PHOUE-POSS (a), PHUE-O3 (c) and corresponding PS controls (b and d) after 7 days.](image)

**Fig. 5.5:** Fluorescence microscope images of live/dead-stained mouse connective tissue fibroblasts on PHOUE-POSS (a), PHUE-O3 (c) and corresponding PS controls (b and d) after 7 days.

The results of the hydroxyproline assay for PHOUE-POSS showed that the control had again a significantly higher hydroxyproline concentration than the coating (Fig. 5.4). Hence, the functionality of fibroblasts on untreated and modified PHA coatings seemed to be reduced. Surprisingly, the POSS derivatisation of PHOUE did not improve cell adhesion, proliferation and collagen production considerably. For PHUE-O3, the results of the hydroxyproline assay showed a reduced hydroxyproline concentration, but they were not very significant because the error of the control was big (data not shown).
The ozone derivatisation drastically decreased the water contact angle of the surface and increased the cell adhesion compared to the untreated PHUE. In parallel to this, the cell proliferation was clearly decreased. The PHUE-O3 coating showed similar properties to PS optimized for cell culturing, if the results from the hydroxyproline were not considered.

According to our results, the PHAs can be divided into two groups according to their suitability for cell attachment, proliferation and function:

PHOUE, PHOUE-POSS and PHUE-O3: These biomaterials provided good cell attachment regarding the live/dead staining. The cell proliferation was equal or better on these coatings than on PS. The collagen production was reduced with these PHAs.

PHUA and PHUE: Neither coating provided very good cell attachment (live/dead stain) and most of the cells became dislodged during the staining procedure. Both coatings supported proliferation resulting in cell numbers higher than on the PS control. The cells produced lower amounts of collagen on both coatings in comparison to controls.

5.4 Conclusions

Cytotoxicity experiments as well as cell-culturing experiments on PHA coatings showed that biosynthesised mcl-PHA with different chemical compositions are suited for medical applications, when an effective purification procedure such as repeated dissolution-precipitation was applied. Cell adhesion was limited on the non-polar surfaces of mcl-PHA and hence a derivatisation of the PHA surface was required to improve its hydrophilicity and to optimise the cell attachment. For long term applications the type of chemical modification may be of crucial importance. Surface-modified PHAs like PHUA-O3 could loose the polar groups during degradation and consequently the surface would become more hydrophobic. Homogeneous PHAs on the other hand will not change their surface polarity drastically during degradation. Possibly, the cleavage of ester bonds during degradation will increase the surface polarity through the formation of additional carboxyl and hydroxyl groups. Hence, long time studies are needed to elucidate these questions. For applications where
cell attachment has to be avoided, mcl-PHA with saturated side-groups could be well suited.

Our measurements indicated that the collagen production of fibroblasts was reduced on all PHA coatings, compared to the cells on PS. Hence the functionality of fibroblasts on PHA coatings appeared to be reduced. The reason for this observation was indistinct and further measurements with another assay are needed to confirm this result. Furthermore the collagen type should be determined to identify eventual changes.
Chapter 6

General conclusions
The use of polymeric materials for the administration of pharmaceutical and as biomedical devices has increased dramatically over the last ten years (Shikanov et al. 2005). Important biomedical applications of biodegradable polymers are in the areas of drug delivery systems and in the form of implants and devices for fracture repairs, surgical dressings, artificial heart valves, vascular grafts, and organ regeneration. It has been concluded that PHA has the potential to become an important compound for biomedical applications (Valappil et al. 2007; Williams et al. 1999; Zinn et al. 2001) and the results of this thesis further confirm this conclusion.

The aim of this work was to adapt the properties of mcl-PHA for medical applications by optimising its fermentative production and downstream processing (chapter 3 and 4). PHO was used as a model for the class of mcl-PHA. To analyze and optimize the production of mcl-PHA, a new method for the quantitative analysis by gas chromatography (GC) was developed (chapter 2). Finally, the biocompatibility of mcl-PHA films was investigated in vitro using mouse fibroblasts and the polymers were chemically modified to enhance their properties (chapter 5).

**Quantitative analysis of PHA**

Nuclear magnetic resonance spectroscopy (NMR) is a common tool to analyse and quantify bacterial PHA. But NMR analyses are restricted to pure PHA and are very time-consuming, whereas GC measurements are cheap and need less time. Established methods for the transesterification of PHA in biomass with protic acids were shown to be inadequate for the quantitative analysis of mcl-PHA due to an excessive formation of side-products. This problem could be solved by using the Lewis-acid BF$_3$ in water-free methanol. However, the development of a fast method that can be used to derivatise and analyse PHA-containing biomass in a short time (e.g. 1 h) remains a challenge. For PHB-containing biomass the derivatisation time was drastically decreased to about 4 minutes by using microwave heating (Betancourt et al. 2007). Presumably, microwave heating can likewise be used for the accelerated transesterification of mcl-PHA. Alternatively, other derivatisation reactions like the reductive depolymerisation by LiAlH$_4$ could be applied to mcl-PHA. Ideally, the PHA concentration in a fermenter is measured continuously in situ, but a reliable method has not been developed yet.
**PHA biosynthesis – possibilities to reduce the endotoxic contamination**

P-limitation was successfully applied in this work to the fermentative production of mcl-PHA to reduce the endotoxic contamination. The confirmation of the structural change of lipid A was unsuccessful and it would be interesting to investigate it. However, P-limitation could not be extended arbitrarily in the chemostat as the cell growth rate became restricted. Possibly, P-limitation would be more efficient in a two-stage fed batch or two-stage continuous fermentation with P-limitation in the second stage. In this case the endotoxic activity would only decrease if the structure of Lipid A is changed during P-limitation. Recent experiments with *Francisella novicida* showed that certain microorganisms own specific phosphatases for lipid A (Wang et al. 2004; Wang et al. 2006). If similar phosphatases were also present in *P. putida*, the phosphorylation of lipid A could be modified during the adaptation of cells to altered culturing conditions. Otherwise, the genes coding for these phosphatases (LpxE and LpxF) could be overexpressed in PHA producing bacteria like *P. putida* to detoxify the lipid A, independently from culturing conditions.

Another alternative to avoid the contamination with LPS is the production of PHA with Gram-positive bacteria. PHAs were produced with the genera *Bacillus* and *Streptomyces* (Valappil et al. 2007). However, the ability to synthesize mcl-PHA with Gram-positive bacteria appears to be rather limited (Valappil et al. 2007).

**PHA recovery**

PHA extraction with organic solvents and precipitation with non-solvents results in a polymer of high quality, but this standard method is rather complicated and produces large amounts of mixtures of solvent and non-solvent. Temperature controlled extraction and precipitation (TCP) was found to be a good alternative to the classical approach. It could be used to extract and precipitate PHO and co-polymers of PHO efficiently. The recovery of PHA is basically complicated by the fact that PHA is stored intracellularly. It would be simplified a lot if the PHA granules were secreted to the medium. The cells could be separated from the supernatant by centrifugation and the PHA granules would be recovered with the supernatant in high purity. However, the transport of huge PHA granules through the membrane of intact cells appears to be unrealistic. Recently, the production of extracellular PHA with *Alcanivorax borkumensis* SK2 has been claimed (Sabirova et al. 2006), but it was not shown that extracel-
lular PHA did not originate from lysed cells. Hence, serious doubts remain and further research is needed.

**Towards biomedical applications**

Cytotoxicity experiments with aqueous extracts from mcl-PHA as well as cell-culturing experiments on films of mcl-PHA confirmed that these polymers were biocompatible when mcl-PHA of high purity was used. Cell adhesion could be improved by chemical treatments of the non-polar PHA surface. However, the functionality of mouse connective tissue fibroblasts was limited. The reason for this observation remained obscure and further investigation is needed.

An important but restraining aspect is the processing of mcl-PHA. For most mcl-PHA processing is difficult due to their elastomeric and sticky properties. Hence, appropriate processing techniques have to be developed for a successful commercial application. Again, chemical derivatisation may help to increase the crystallinity of PHA, thereby facilitating its processing.

The results obtained in this doctoral thesis show, that optimized fermentation and downstream processing generate mcl-PHA of high quality with very low endotoxic contamination that can be used in the biomedical field. The broad range of different mcl-PHAs combined with chemical modification allows adjusting the chemical and physical properties to the target application. Thus, a basis for successful biomedical applications of mcl-PHA was established with this work.
References


References


### Curriculum Vitae

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1976</td>
<td>Born in Bern, Switzerland</td>
</tr>
<tr>
<td>1991-1996</td>
<td>Grammar school, Bern</td>
</tr>
<tr>
<td>1996</td>
<td>Matura type C</td>
</tr>
<tr>
<td>1996-1999</td>
<td>Chemistry studies at ETH-Lausanne</td>
</tr>
<tr>
<td>1999-2002</td>
<td>Chemistry studies at university of Basel</td>
</tr>
<tr>
<td>2002</td>
<td>Diploma in chemistry, with physics as minor subject</td>
</tr>
<tr>
<td>2002-2003</td>
<td>Trainee in polymer analytics, Ciba specialty chemicals Basel</td>
</tr>
<tr>
<td>2003-2007</td>
<td>PhD studies in bioprocess engineering at Empa St. Gallen and ETH Zurich</td>
</tr>
<tr>
<td>2008</td>
<td>Project manager at Sika Technology</td>
</tr>
</tbody>
</table>