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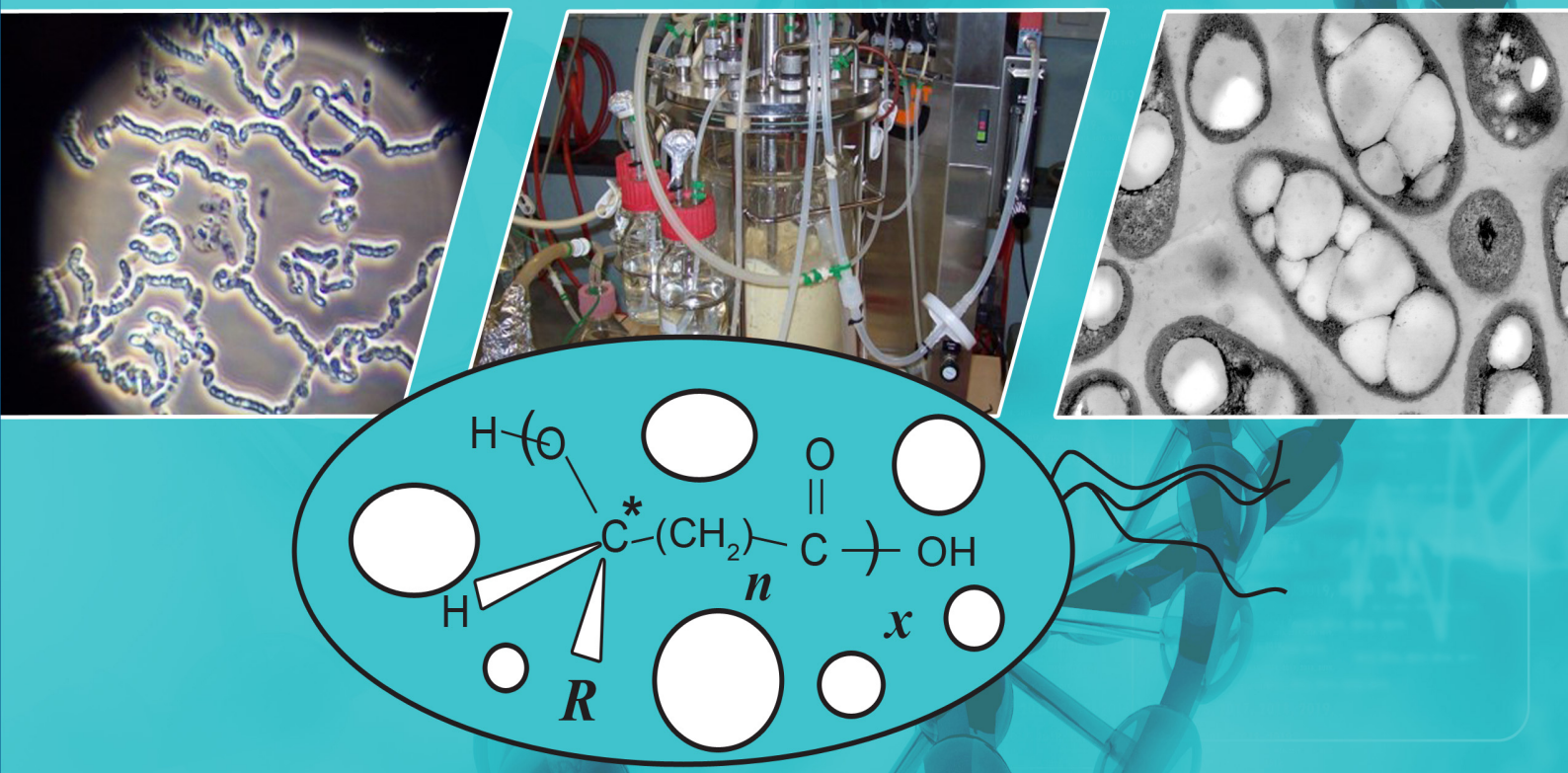
Microbial Biopolyester Production, Performance and Processing

Bioengineering, Characterization, and Sustainability



Editor:
Martin Koller

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(Volume 2)

Microbial Biopolyester Production, Performance and Processing

Bioengineering, Characterization, and Sustainability

Edited by:

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FOREWORD

I remember my first experience with PHA sometime in the middle eighties of the last century, when Professor Anton Blazej, rector of Slovak Technical University, visited ICI and handed me over about 20 g of this material. The slabs looked as remnants from specimens for impact testing. He strongly recommended to get more information on the material and perhaps to start some research with this "plastics of the future". I was rather deeply involved in material sciences, aiming mainly on polymeric multiphase systems, such as blends and composites and appropriate procedures of their modification to get particular properties for specific applications. That time the basic polymer for me was polypropylene; concerning biopolymers, we carried out some marginal investigations on mixtures of starch with polyethylene. However, the amount of PHA was too small to perform even preliminary material testing; in addition, no information on the composition was provided, so we just accepted it as an interesting but still "exotic" material. However, I could not continue ignoring the PHA for long time since, in 1991, Dr. Hanggi, the owner of the company Biomer, Germany, addressed me with the request to make some measurements of mechanical properties on PHB which Biomer was producing and selling on commercial basis. He provided more or less unlimited amount of material (considering laboratory scale of research), so, besides the tests for Biomer I could really start to play with the PHB in the lab.

Biodegradable plastics started to become my main research topic at the end of the nineties, when Stanislav Miertus, professor at ICS UNIDO Trieste (my former colleague at Polymer Institute SAS in Bratislava, where we worked at the same time on our PhD thesis) invited me to Trieste for a workshop on BDPs and asked me to give a short lecture on BDP´s situation regarding research and application in Slovakia. He was surprised when I offered a full lecture on our research on modification of PHB. That time, Miertus was organizing a set of workshops under UNIDO auspices, and supported the idea to promote BDPs by collecting a number of scientists including top researchers in this area. Since I was honoured to be invited as member of this group, within this scheme, I had a unique opportunity to meet and discuss all aspects of BDPs with leading experts in this field, such as Gerhart Braunegg, Emo Chiellini, Ramani Narayan and number of others. Moreover, we had the opportunity to visit various countries (each workshop was supported by UNIDO but organized by local organizers in a particular country for a selected region), and to get familiar with various attempts and attitudes to contribute to the development of applicable BDPs.

The research and promotion of PHAs was one of the most important topics discussed in this expert pool. Within the group, all relevant aspects concerning BDPs were considered with the main concern devoted to PHA and PLA. Generally, it was agreed that to achieve a substantial

increase in production of BDPs, the materials must be suitable for high volume applications; packaging materials were considered as the most prospective goods since biodegradability could be of great advantage offering an alternative way to current waste management to handle the vast and continuously growing amount of plastics garbage. However, this market was occupied by polyolefins and PET. To be competitive, two basic conditions had to be met. The BDPs should have the ultimate properties as well as processing parameters similar to currently used materials and the price should approximate the cheapest variants of petroplastics. That time it was believed that if BDPs would have suitable properties, a price approximately double of polyethylene would be acceptable, taking into account simpler waste management logistics and also positive response of the public regarding the environmental feasibility. However, a vicious circle appeared due to the fact that PHA and PLA seemed to be too expensive for high volume application and, consequently, because of low consumption and low production amounts on the pilot scale level, the price remained high.

The aspect of price of PHAs was generally tackled by research aimed for preparation and effective production of PHB and related copolymers, where the main concern was to make the prospective production cheaper. To achieve this, various substrates were investigated, mainly carbonaceous sources of waste. An example of such approach was represented by the project WHEYPOL, granted by the 5th EU Framework Programme, coordinated by Gerhart Braunegg. The goal was to produce PHB from whey available in large quantities as a waste stream from production of cheese. The project was successful and the major outcome was the design and setup of complete production facility for production of PHB with capacity 20,000 tons per year. This amount was considered as optimal from point of view of initial investment needed and final cost of the polymer being competitive enough. The plan was to build up a pilot production plant with a capacity of 800 t per year which could produce commercial material but the main goal was to prove that the technology is feasible also on industrial scale. Unfortunately, the proposal submitted to EC for the aspired demonstration project was rejected with reasoning that for this type of projects no demonstration projects are supported. (In this context, recently I saw statistics data on the ratio of budget for research from public resources, showing that in USA the ratio between basic research, applied research and demonstration was 24/28/48, in China 11/32/57, which is in huge contrast to the figures for the EU: 92/2/6 http://youtu.be/oy_wxg5-nyY. Obviously, significant number of results of European research paid from EU sources is applied outside the EU region). Consequently, private investors were considering to take over, even an investor from Slovakia was quite keen to invest 2 million EUROs needed for the pilot plant construction. Gerhart Braunegg and Martin Koller acted as consultants in discussions regarding the optimal technology and especially feasible substrates for growth of bacteria and selection of powerful microbial species. Unfortunately, before reaching to an acceptable conclusion, the financial crisis knocked all plans on the head, and the investor was happy to be able at least to save his

current business running.

The second strategic aspect in promoting the development of BDPs consisted in properties, both ultimate and regarding aspects of their processing. The main problem with ultimate properties is represented by rather high Tg of PLA (about 60 °C) and although the value for PHB is below RT (around 10 °C), still both materials are extremely brittle. Moreover, both (especially PHB) suffer from quite pronounced physical ageing leading to lowering the toughness; this makes the material even more brittle within a period of few weeks of storing. Regarding processing, the main problem was that at processing temperature of PHB being at least 180 °C or more, thermal degradation *via* hydrolysis proceeds too fast. Steep decrease of molecular weight was reported, contributing to further increase of brittleness; certain auspicious technologies, especially film blowing were impossible to be applied.

However, at the beginning of this millennium, the situation began to change. Globally, a number of research teams have been interested in biodegradable compostable plastics, important achievements were reported and patented dealing with all aspects which prevent high volume application of BDPs. Moreover, industry started to seek the potential of compostable plastics for packaging and real commercial applications appeared first in small volume but gradually the production was increased. A substantial contribution to this trend was launched by starting production of PLA by Cargill with a capacity 150,000 tons per year, as a first really high volume production of a compostable, biodegradable polyester. Today, production of biodegradable compostable plastics is claimed to be raising by 20 % per year. In spite of this the portion of biodegradable plastics in overall plastics world production is only around 1 %.

While at the beginning of the millennium, the main advantage of BDPs concerning the environmental effects was seen in biodegradability and prospective compostability, recently new attitude is stressed, namely biobased origin is claimed to be the most valuable environmental contribution. This trend is related to carbon dioxide production as a reason for global warming. Materials based on renewable resources are defined as "CO_2 neutral", it means that the same amount of CO_2 consumed for creation of the renewable organic raw material is released at the end of lifetime of the product, regardless on the way of the waste disposal. Thus, the classical plastics (mainly petrobased) are adjusting to new environmental trends by creating biobased polyethylene or poly(ethylene terephthalate). The compostability would by such a way be considered as less important, although still useful when considering the application of new environmentally friendly plastics.

However, in the past the scientific projects were aimed almost entirely to scientific and technological development, recently more concern is devoted to broader goals, where a significant part of work (and funds) is aimed to networking, seminars for companies and

dissemination to a broader public (including TV and radio); in this kind of projects scientific research is performed mainly as case studies. Even under this scheme I was a partner in an ERDF project PLASTiCE (coordinated by Andrej Kržan who authored chapter 2 of this *e*Book volume) where, besides others, also a biodegradable blend based on PLA and PHB was successfully tested for production of packagings for eggs by thermoforming.

Coming to the end I would like to stress two, in my view, important non-scientific aspects. The first one is related to the impatience of both scientists and industry to apply the new, apparently valuable, results as soon as possible. If the product is not sufficiently tested, *i.e.*, it passes the standard tests, but the conditions of utilizations as well as disposal issues are not addressed in all details, it may be possible the product fails under certain circumstances which were not considered to be crucial. Such failure will probably disqualify the product from further utilization but, at the same time, the public extends the failure to the whole group of novel, biodegradable plastics. By such a way, an irresponsible application may create opposition and retard the development of many useful environmentally sound solutions.

The other comment is aimed to compostable plastics which may turn to be a valuable alternative to the current technologies of waste management. To design excellent biodegradable compostable plastics in the lab and to develop a suitable technology is just the very first step. It has to be realized that from the environmental point of view, "biodegradable" and "compostable" *per se* means nothing. What really counts is the product which, after passing its lifetime, is *de facto* "biodegraded" and "composted". To complete this task, the full value chain must be designed from commercial production of materials and industrial production of goods up to collecting waste, sorting and transporting it to composting plants, which, of course, must be available. An important part of this line is also certification and labeling of compostable, biodegradable and/or biobased materials and products so that the collection and sorting of waste would be easier. Establishment of a certification scheme in Slovenia and Slovakia was also a goal accomplished within the PLASTiCE project mentioned above.

In my view, the current situation for biodegradable plastics looks promising. It seems that all questions related to application of compostable materials are defined, many are fully or at least partially answered. Concerning areas where, up to now, research efforts did not point out clearly promising way, degradability of plastics in marine environment should perhaps be mentioned as the most important challenge. Regarding biodegradable polyesters, especially PHAs and PLA, considering the above detailed current concerns, both are biobased and their compostability is an important extra bonus, so that new materials for various applications are

applied in growing volumes, industry indeed is interested to participate in this area and response of the general public is generally highly positive.

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PREFACE

This *e*Book presents current R&D activities accomplished in diverse research institutions worldwide aiming at replacing well-known plastics originating from fossil resources by alternatives from nature. The importance of these R&D endeavors can be visualized by considering a global production of 300 Mt of plastics per year; the major share thereof is recalcitrant against bio-mediated degradation, and a rather modest portion of only about 20% of spent plastics undergoes recycling. “Environmentally degradable plastics” (EDPs) as sustainable alternatives are available, but cover less than 5% of today´s plastic market. Further, the expression “environmentally degradable plastic” is often used highly ambiguously or even in an explicitly incorrect context; so called “bio-plastics” do very often not come up to the characteristics “biobased”, “biodegradable” or “biocompatible”.

Microbial polyhydroxyalkanoates (PHA), a versatile group of polyesters produced by nature as prokaryotic storage materials, are contemporarily attracting increasing attention as polymeric basic materials to be implemented in various fields of the plastic market. This fact is manifested by the nowadays exponentially growing number of scientific publications and patent applications related to this topic. Not only does the biobased nature of PHAs underline their significance for sustainable future developments, but also the high flexibility and adjustability of their properties, their biodegradability and, to a steadily increasing extent, their biocompatibility. To develop overall sustainable and efficient production processes, one has to take into account all single process steps, starting from microbiology and enzymology, selection of suitable inexpensive raw materials, optimizing process engineering and process regime, until enhancement of product recovery in terms of time-, energy-, and material input.

As mentioned before, we nowadays unambiguously witness a tremendously increasing trend towards new polymeric products from nature to replace contemporary plastic based on fossil feedstocks, especially in areas where established, highly recalcitrant plastics cause environmental concern. After now already decades of global R&D developments in the field of microbial polyesters (mainly polyhydroxyalkanoates - PHA), and numerous body blows on the way to their since a long time announced market penetration, it is generally recognized that the success of these promising new materials needs the improvement of both material performance and production prices. The perception of our exigency to exit today´s disastrous “Plastic Age” were my incentive for about fifteen years of research in the field of these biopolyesters.

Starting with my Master thesis on novel, ecologically benign methods for extraction of PHA from microbial biomass in the group of Gerhart Braunegg at Graz University of Technology in the year 2000, I got more and more fascinated by these unique biopolyesters. Not only the

multiple and complex metabolic roles, the various functions of PHA in diverse ecosystems, the high diversity of PHA accumulating microorganism and the inestimable variety of different "plastic like" properties of different types of PHA attracted my attention, but also the variety of cultivation strategies in discontinuous and continuous mode which can be implemented to produce PHA in an efficient way at pre-defined composition and quality. Most of all, raw material aspects which need to be addressed to make PHA production cost-efficient for me always constituted the central field of interest; in this context, I had the honor to perform my doctoral thesis, guided by G. Braunegg, on production of PHA from whey as an abundant raw material in the frame of the EU-FP5 project WHEYPOL. Years later, we upgraded another industrial surplus material to a feedstock for PHA biosynthesis, namely waste lipids from the rendering and animal processing industry. Here, enabled by the EU-FP7 funded project ANIMPOL, our multidisciplinary and international consortium demonstrated how the utilization of this waste stream on the one hand contributes to minimizing waste disposal issues, and, on the other hand, provides for cost-efficient production of PHA biopolyesters and even positively contributes to biodiesel industry. As a common feature of all PHA-related research activities I carried out together with G. Braunegg, one should emphasize the strong industrial embedding in all projects, either as direct project sponsors, advisors, or as active project partners. At this point, I would like to take the chance to express my graduate to all our industrial partners and financiers who enabled our research and, by cooperating with us, underlined the high industrial significance of this novel class of biomaterials.

Coming to the core part of this issue, all process phases, starting from the genetic and enzymatic procedures in the microbe´s interior, raw material selection and allocation, the production process in adequate bioreactors including kinetic analysis and mathematical modelling, process engineering aspects regarding fermentation modes, feeding regimes and bioreactor selection, and, last, but not least, product recovery and refining ("downstream processing") need consideration and optimization; this goes in parallel with market issues and holistic consideration of sustainability aspects.

Although an increasing number of high-quality monographs and reviews are contemporarily published, none of these addresses all aspects of the holistic PHA production process in a comprehensive way. The volumes *"Microbial biopolyesters – Production, Performance and Processing aspects 1 and 2"* of the *e*Book series "*Recent Advances in Biotechnology*" addresses this shortcoming by sixteen articles written by carefully selected leading scientists and their associates, performing their work on three different continents. Of course, it was connected to considerable efforts to select the most outstanding groups for all special chapters. These research groups are since years or sometimes even since decades active in different scientific areas involved in this fascinating and strongly emerging field of research,

as demonstrated by their tremendous number of related publications and patents. I am outstandingly happy and thankful to all these scientists for their willingness to spend their precious time in contributing to the work at hand. As a result, the synopsis of both volumes is an outstanding composite of comprehensive articles covering all single aspects which are nowadays recognized to be decisive for sustainable and efficient production of microbial polyhydroxyalkanoate (PHA) biopolyesters.

The volume at hands, *"Part 2: The Engineer´s Point of View (Aspects of Engineering, Characterization, and Sustainability)"* encompasses eight specialized chapters addressing topical endeavors to enhance the processibility of PHA towards vendible bio-technomers, give novel insights into sustainability aspects of PHA production as a holistic process, and offer critical insights into the features which contemporarily need to be addressed to make PHA competitive with their counterparts of petrochemical origin in terms of economics and material performance. These chapters address market issues needed to accelerate the success of PHA and related biopolymers on the market, elaborates sustainability issues, and provides an insight into the impact of PHA composition on the monomeric level on the final properties of PHA biopolyesters. In this context, the reader will learn about current techniques for tracing intracellular PHA, and for characterization of these polyesters regarding their decisive physical properties. Further, new strategies are presented how to increase the quality of PHA by adjusting the degree of crystallinity and profiting from nanotechnology to design smart biopolymers. More engineering-related chapters provide the up-to-date knowledge on bioreactor design and fermentation regime to enhance PHA production by innovative technological means, or emphasize the need for sustainable downstream processing techniques for PHA recovery from microbial biomass. Finally, mathematical modelling as a tool to better comprehend PHA production and to restrict the number of laboratory experiments are discussed.

The subsequent paragraphs shall give the respected readership an impression of the contents of the specialized chapter articles of this issue:

CHAPTER 1:

Lucia Amaro and colleagues, active at the highly recognized laboratories of Emo Chiellini in Pisa, provide an analysis of the main criteria for the selection of (bio)materials suitable for the production of flexible and rigid packaging which addresses the expectations of the customer, economics and environmental aspects. In this context, the authors focus their critical paper on mechanical properties, melt flow behavior of PHA homo- and copolyesters, processing by melt extrusion, film blowing, injection molding, *etc.*, and, as culpably underestimated in the past, on odor issues of biopolymers. Guidelines to overcome current problems faced by bacterial polyesters and their relationship with the current polymers market are also presented

in this chapter.

CHAPTER 2:

Andrej Kržan from the National Institute of Chemistry in Ljubljana, Slovenia, demonstrates in his article that EDPs such as PHA are growing rapidly in number and regarding their fields of application. The author stresses that assessing their major characteristic, namely degradability, which also includes biodegradability as final stage, is a challenging issue both scientifically and in technological aspects, and, in the past, resulted in different interpretations. To standardize criteria and techniques, various standards were created by different standardization institutions, which also act as a basis for certification patterns. An up-to-date inventory of the rapidly growing standardization body is presented with basic interpretation to help guide the non-expert.

CHAPTER 3:

Predrag Horvat and colleagues from the University of Zagreb, Croatia, are well-known in the scientific community for their expertise in mathematical modelling of bioprocesses. In their comprehensive article, these authors emphasize that process/strain optimization procedures are unavoidable in the improvement of large-scale bioproduction processes. These procedures are necessary on bioreactor level and on biochemical/genetic level of producing strains. Here, mathematical modeling is described as a useful remedy. Mathematical models applied for PHA biosynthesis (classified as both structured and unstructured), are classified into low-structured, formal kinetic, high-structured (metabolic), dynamic, neural networks, cybernetic, or hybrid model type. In their chapter, the authors discus each specific group of models is in the light of its applicability and benefit for increased productivity, enhancing of the specific PHA biosynthesis rate, and better understanding of the intracellular metabolic regulation systems. Characteristics of production strains, particularities of MMC and features of industrial-scale plants cannot be described by one sole model type alone able to address all different needs. In this context, the authors suggest that satisfying, compromised, perhaps most promising solutions are obtained by the combination of mechanistic, neural, cybernetic, and computational fluid dynamics (CFD) types of models. This hybrid modelling approach generates a holistic picture of the PHA production process by benefiting from the advantages of different model types.

CHAPTER 4:

Kianoush Khosravi-Darani and colleagues, located at the Shahid Beheshti University of Medical Sciences, Tehran, Iran, summarize engineering issues in PHA production by linking bioreactor design to biochemical ongoings in PHA producing strains. The authors emphasize

that addressing the effect of the hydrodynamic and mass transfer phenomena on PHA production is necessary independent of the applied bioreactor types. PHA production performance increases with operational and design parameters such as superficial gas and liquid velocities and also height and length of the bioreactors in the accumulation phase. Time constant analyses substantiate previous findings that the rate of oxygen consumption constitutes the process-determining step at such superficial gas- and liquid velocities exceeding the specific values. Batch, fed-batch and continuous fermentation mode are compared regarding cell concentrations and intracellular PHB mass fractions in bioreactors to reduce production expenses. Further, the authors describe that the control of carbon to nitrogen ratio has direct impact on PHB production, and accumulation of PHB inclusion bodies in most microorganisms depends directly on limitation of nutrient components. Application of recombinant or wild microbial production strains which produce PHB during balanced growth could enable the setup of highly efficient one-stage continuous chemostat production systems. In addition, the authors describe autotrophic cultivation with CO_2 and CO as another strategy towards cost-efficient PHA production.

CHAPTER 5:

Abhishek Dutt Tripathi from the Banaras Hindu University in India and his associates devote their chapter to the discussion of techniques for downstream processing to recover PHA from microbial biomass. This is of special importance due to the fact that downstream processing constitutes the second largest cost category in the PHA production cycle. The authors compare various established and novel recovery techniques reported and studied at small laboratory scale as well as in (semi)industrial facilities. These techniques encompass methods of solvent extraction, chemical biomass disintegration, enzymatic and/or mechanical disruption, application of supercritical fluids, flotation, subjecting biomass towards gamma irradiation, and implementation of aqueous two-phase systems. The article summarizes all currently known recovery methods, and compares them in terms of efficiency and the quality of the resulting PHA regarding product purity and impact of the recovery technique on molecular masses.

CHAPTER 6:

Giin-Yu Amy Tan and co-authors, associated with Jing-Yuan Wang and Swee Ngin Tan, constitute an authorship distributed over the Hong Kong Polytechnic University and the Nanyang Technological University in Singapore. These authors address current and emerging advanced analytical technologies for biopolyesters characterization, a definitely highly topical item. The authors emphasize that analysis and characterization of PHA biopolyesters remain an integral aspect of the PHA industry, correlating the microbial processes performance and PHA's physiochemical properties. The vast number of structurally diverse PHA monomers

has also made PHA analysis extremely challenging. Numerous techniques have been exploited for the detection, quantification, and characterization of microbial PHA. New techniques are also continuously being developed with advancing instrumentation capabilities. This book chapter introduces the basics underlying current and emerging PHA analytical techniques, and summarizes key protocols and information related to these techniques. The potential applications of emerging techniques are also highlighted and discussed.

CHAPTER 7:

The complexity of PHA composition on the monomeric level is also addressed by the article provided by Ayaka Hiroe, Sho Furutate, Shoji Mizuno, and Takeharu Tsuge from the Tokyo Institute of Technology. These authors focus on the recent progress in improving two types of PHA, namely 3HB-based copolymers and unusual PHA homopolymers, which show improved material properties and/or cannot be synthesized in nature. 3HB-based copolymers which not only include 3-hydroxyvalerate, 3-hydroxyhexanoate, and long chain 3-hydroxyalkanote-containing copolymers, but also branched building blocks like 3-hydroxy-4-methylvalerate or 3-hydroxy-2-methylbutyrate, aromatic building blocks like 3-hydroxy-3-phenylpropionate, 3-hydroxyphenylvalerate, or 3-hydroxy-5-(4′tolyl)valerate, and even lactate-containing copolymers are reviewed in this chapter. On the one hand, the work discusses material properties like melting points and glass transition temperatures of these different biopolyesters in dependence of the structure and molar share of different building blocks; on the other hand, also the metabolic background of the biosynthesis of these rather exotic PHA constituents are elucidated.

CHAPTER 8:

It is well known that the physical properties of a thermoplastic semi-crystalline polymer such as thermal, mechanical and gas permeability properties are markedly influenced by the crystalline structure and morphology. As described by Patrizia Cinelli and associates from the University of Pisa and the National Research Council in Pisa, Italy, these characteristics are controlled by the solidification and processing conditions. A full knowledge of the structure and morphology of the crystalline phase and its quantification are crucial for both the comprehension and the prevision of the final properties of a PHA-based material, as small changes in crystallinity can dramatically modify its properties. The principles that govern the relationships between structure and properties of the biopolymers are the same that determine the behavior of the fuel-derived polymers. The authors underline that extensive use of PHA has been hindered until now by some insufficient properties relevant for practical applications, which make these polymers not equivalent to conventional thermoplastics; many attempts have been performed to improve their properties, mainly by preparing mixtures with

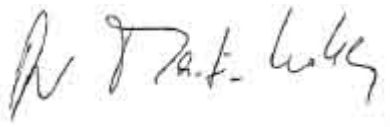
various different compounds or with other polymers. In the chapter of Cinelli *et al.*, an analysis of the mechanical properties, especially related to the crystalline degree, of PHB, some of its copolymers, blends, composites, and nanocomposites are discussed.

With this issue, we wish to address scientists active in the field of biopolymers, fermentation technology, and polymer science. The *e*Book is also dedicated to students of higher level which are involved in the fields of polymer chemistry, environmental science, biotechnology, microbiology, and overall life sciences; we hope that this *e*Book is helpful for you! In addition, we strongly believe that the issue attracts also the attention of representatives of polymer industry. Do you want to get to feet on the ground of innovative polymeric products? This might provide for the ignition sparks for a broader implementation of PHA biopolyesters on industrial scale.

I am tremendously optimistic that the exploratory and scientific efforts collected and summarized in the *e*Book volume at hand will motivate researchers all over the planet to deepen their R&D activities in this field, and to attract the interest of undergraduates as well as of innovative representatives from relevant industrial sectors. First and foremost, these activities shall boost the impatiently desired market penetration of such types of "bio-plastics" which fairly merit this designation. The content of this *e*Book emphasize that "bio-inspired" remedies for prevalent ecological problems are already available, developed by experts engineering and life sciences, or these solutions are at least being developed; they are expecting their industrial implementation in the emerging field of "White Biotechnology"!

Again, I would like to cordially thank all contributors to this issue for their supreme work.

Graz, Styria, Austria, January 25th, 2016.



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CHAPTER 1

Bacterial Polyesters: The Issue of their Market Acceptance and Potential Solutions

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Abstract: The present book chapter is aimed at providing an outlook about the synthesis, properties and potential applications of bacterial Poly[(*R*)-3-hydroxybutyrate)] (PHB) and its copolymer with (*R*)-3-hydroxyvalerate (PHBHV). Selected reports were analyzed in order to provide strategies aimed at producing standard formulations based on PHB and PHBHV by given emphasis on packaging applications. Modification of PHB and PHBHV properties by using reinforcing agents and blends with biodegradable and non-biodegradable polymers has been reviewed. The strategies for modulation and/or standardization of PHB and PHBHV, in composite formulations and blends, were done by taking into account thermo-mechanical properties, dimensional stability and melt flow index data. Odor control in the end-products by using additives and potential methodologies to mitigate it as well as guidelines for the production of odorless, standard and biodegradable PHB and PHBHV based products for packaging were also provided.

Keywords: Application, Bacterial polyesters, Blends, Composites, Dimensional stability, Market, Melt flow index data, Nanocomposites, Odor control,

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Polyhydroxyalkanoates (PHA), Poly(3-hydroxybutyrate) (PHB), Processing, Standardization, Thermo-mechanical properties.

INTRODUCTION

The world plastic production has risen in the recent years from 299 million tons in 2013 to 311 million tons in 2014, representing a 3.9% increase. On the other hand, in Europe, the trend has been more stable after the 2009 downturn. The demand for plastics in 2013 in Europe was 46.0 million tons, an increase of 0.1% in comparison to 2012. Packaging, building and construction represent by far the largest markets, followed by the automotive, electrical and electronic industries [1].

Poly(lactic acid) (PLA), poly[(*R*)-3-hydroxybutyrate)] (PHB) and its copolymer with (*R*)-3-hydroxyvalerate (PHBHV) are the best known representatives of Poly(hydroxyalkanoates), PHAs family [2]. These polyesters have attracted widespread attention as environmentally friendly polymers, which can be used in a wide range of agricultural, industrial, and medical applications. PHAs can vary substantially in composition, as there are over 150 known constituents, resulting in an enormous diversity of material properties [3]. Given the overwhelming diversity of bacterial polyesters and based on our experience on bacterial polyesters the present chapter focuses on PHB and its copolymers with 3-hydroxyvalerate (PHBHV) as the most representative members within the spectrum of bacterial polyesters.

Considering the biodegradability and thermoplastic characteristics of PHB and PHBHV, it is reasonable to propose these bacterial polyesters as good candidates to replace plastics derived from non-renewable sources, for example low density polyethylene (LDPE) and Polypropylene (PP). PHB and PHBHV could be capable of replacing LDPE in applications represented by large market consumption and, in which the compostability could represent a good strategy for the disposal of the finished product. Packaging, for example, represents the application where bacterial polyesters can be used directly or formulated for the production of rigid and flexible products, for a wide variety of markets (*i.e.*, electronic, cosmetic, pharmaceutical and food). In Europe, packaging is the

largest application sector of the plastic industry and represents 39.6% of the total plastic demand (www.plasticseurope.org, 2015). However, due to the high prices of bacterial polyesters (ranging from 4.5 to 5.5 €/kg) compared with the price of a poly(ethylene) resin (1.6 euros/kg), their particular odor and their low reliability into reproducing the properties needed for a specific application (*i.e.*, film production), bacterial polyesters are now facing low market acceptance. Moreover, the global production of bacterial polyester, around 100,000 tons per year [3], is not enough to gain a market share in packaging sector, thus confining the bacterial polyesters into niche sectors as for example agricultural (with a 3.4% of European demand in 2014) (www.plasticseurope.org) in which the biodegradability could be the differentiation factor.

The odor and the lack of property robustness of bacterial polyesters are mainly due to their particular synthetic route, which is based on bacteria that synthetizes them as an energy feedstock reserve. Namely, polymers synthesized by microorganisms and stored within the cells in response to certain nutrient conditions in the fermentation media.

This fact renders the bacterial polyesters so challenging materials. Scientists, producers, transformers, or whoever wants to use these polymers for any application must adapt their own needs through their direct manipulation. The modification of the molecular weight distribution, the control of the chemical composition and the stereochemistry of the polymer chain, and the insertion of branches and functional groups are until now fully or partially limited when a bacterium does the synthesis in contrast to man-made synthetic routes. Changes in the precursor substrate on the bacterial culture during the PHB biosynthesis is a representative example on the modification of the PHB chemical composition. Recombination of genes of other bacterial species as well as introduction of hydroxyl- and carboxyl containing organic molecules have been until now commonly used for production of (*R*)-3-hydroxybutyrate and (*R*)-3-hydroxyvalerate. These biopolymers are characterized by an increased elasticity and by decreased brittleness, mechanical properties that are preferred for some industrial applications (*i.e.*, film production) [3].

Moreover, from our experience, characterization of certain bacterial polyesters is

CHAPTER 2

Sustainability and Plastics

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Abstract: To fulfill the many expectations placed upon polymers and plastics to remain competitive and acceptable compared to other materials, they must constantly improve in their functional properties and their cost/benefit ratio. More recently it has also become imperative that the environmental burdens caused by polymers and plastics be reduced and that overall sustainability of these materials be raised. Today the environmental aspect of a material are one of the key factors for assessing its acceptability and for making decisions about its use.

Thus there is now a greater emphasis on evaluating the environmental impacts of polymers and plastics. Using the life cycle assessment method polymers and plastics are being compared to each other and to other materials. The environmental emphasis has also prompted active development of new, or in some cases reengineered, biobased and biodegradable polymers and plastics. During the last decade these materials have moved from research laboratories into commercial production and represents one of the fastest growing niche segments in plastics, although the overall quantity still remains relatively low.

Now that bioplastics have been successfully launched and real-life experiences from early applications have been obtained, their sustainability is undergoing a thorough examination. The results show that these new biomaterials cause environmental burdens similar to those caused by conventional plastics. In general, bioplastics offer reductions in emissions of greenhouse gases and the use of fossil resources, whereas the production of bioresources from farming contributes to higher acidification and eutrophication burdens. End-of-life waste management can also strongly influence the overall results. It is expected that future developments in technology and organization

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and the use of second generation bioresources will improve the environmental profile of bioplastics. Bioplastics can contribute to reaching policy goals *e.g.*, regarding the reduction of greenhouse gases but they must be used properly to achieve the desired sustainability benefits.

Keywords: Biobased, Biocompatible bioplastics, Biodegradable, Bioeconomy, Biomass, Bioplastics, Bioresources, Ecobalance, Life Cycle Assessment (LCA), Plastics, Polyhydroxyalkanoates (PHA), Polymers, Standardization, Sustainability.

WHAT IS SUSTAINABILITY?

Sustainability is a politically popular concept that is frequently used in general as well as professional discussions. Despite its omnipresence in our societies very often people are not entirely sure what it really means. The reason for this unusual situation lies largely in the fact that sustainability is a very broad concept with a number of diverse interpretations. It is subject to professional interpretation that may target specific goals, in addition to interpretations by various laymen among an extremely varied general population. Nevertheless, sustainability has become a key decision and opinion-making tool, which may be the foundation for industrial activity, purchasing decisions and policy adoption. Today, synthetic plastic materials generally known as plastics are ubiquitous, very visible, but somewhat poorly understood which leads to relatively frequent questions about their effects, associated environmental burdens and sustainability. Being able to provide accurate and truthful answers to these questions is a key element in defining where, how and which plastics we will use in the future.

Definition

The term sustainable development was first introduced by the World Commission on Environment and Development (Brundtland Commission) in 1987 [1]: *"development which meets the needs of current generations without compromising the ability of future generations to meet their own needs"*. This is the most broadly accepted and quoted definition, however it has little specificity and is in essence more a philosophical guideline than a technical tool. As a consequence, the practitioner is left to find a clearer and more specific definition

that is more amenable to easy application. The common dictionary definition of sustainability is for example: *sustainable - able to be maintained at a certain rate or level* [2]. Rudy Baum coined a nature-based definition directly linked to a prime natural source, saying that sustainability is "Learning to live off the sun in real time" [3]. In very broad terms, one can conclude that what is truly sustainable can be repeated indefinitely, since it has negligible effect on the surroundings. It may be useful to define it by looking at its opposite – an unsustainable process. This is, in the simplest terms, something that can be done once and never again. To give an example, a river mill is sustainable since it can function indefinitely and will in no way affect the source it is using – the flow of the river. On the other hand, mining is a completely unsustainable process – once the ore is removed from the earth it will never replenish itself. This may be a very simplistic definition but it may serve us well due to its clarity.

It is however important to understand that sustainability may come anywhere between these two extreme cases. For example, using a plastic bag several times is better than using it only once, although this is worse than (only hypothetically) using it indefinitely. Assuming that we need to use a bag and would use it in any case, we can use more or less bags depending on our reusing habits. The environmental burdens resulting from the bag production/distribution are directly proportional to the number we use. So, if we were to use every bag ten times we would reduce the environmental burden (from bags) by 90%. Of course using a bag is still worse than not using it, but in life we may need it. This shows that we can be more or less sustainable in our practices without the need to reach absolute extremes. It is important to understand that regardless of the level of sustainability any improvement that we can achieve counts and brings us closer to the desired ideal sustainability.

The Triple Nature of Sustainability

Although we normally associate sustainability with the environment we now understand that a truly sustainable process must take into account three factors: environmental sustainability, economic sustainability and social sustainability. The triple basis is a result of a deeper realization that preserving a pristine environment cannot be equaled to people being able to sustain their lives through

CHAPTER 3

Kinetic Aspects and Mathematical Modeling of PHA Biosynthesis

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Abstract: Process/strain optimization procedures are unavoidable in the improvement of large-scale bioproduction processes. These procedures are necessary on macroscopic (bioreactor) level and on biochemical/genetic level of producing strains. *Inter alia*, mathematical modeling is one of the applicable and useful methods in such procedures. Mathematical models applied for PHA biosynthesis (classified as both structured and unstructured) are denominated as formal kinetic, low-structured, dynamic, metabolic- or high-structured, cybernetic, and hybrid type models, or neural networks. In the chapter at hand, each specific group of models is discussed in the light of its applicability and benefit for increased productivity, enhancing of the specific PHA biosynthesis rate, and better understanding of the intracellular metabolic regulation systems. Characteristics of production strains, particularities of mixed microbial cultures and features of industrial-scale plants cannot be described by a single type of mathematical model since it is not possible to address all the different requirements by a single model type. Therefore, it is more than necessary to fine-tune the modeling approach to each actual case in a sophisticated way. Formal-kinetic and "low-structured" models that are relatively simple and display low computational demand, are applicable for simple cases and are beneficial for mathematical embodiment of "standard microbial cultivations and practices". Hybrid models are used by some

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authors to address certain deficiencies of diverse types of models. In this context, satisfying, compromised, perhaps most promising solutions can be reached if mechanistic, cybernetic, computational fluid dynamics (CFD) and neural models were combined. A hybrid modeling strategy like this generates a holistic representation of solutions for the total PHA biosynthesis process, including all advantages of the different modeling schemes. For example, application of growth media of complex composition usually entails a higher degree of model organization. For the future, really existent biotechnological systems are expected to be expressed by hybrid models of high organization.

Major attention was dedicated to the use of elementary flux modes (EFMs) and yield space analysis (YSA) to develop metabolic models of PHA biosynthesis. The implementation of these methods is reported for numerous case studies which involve modeling of metabolic networks. The chapter concludes with some case studies, where the implementation of EFMs and YSA performed as a powerful modeling tool. It includes the description of intracellular PHA generation and mobilization in the organism *Cupriavidus necator*, the limitation-based picture of the steady-state flux cone of the organism´s metabolic network, the detailed analysis of a multi-stage bioreactor cascade dedicated to continuous PHA production, and metabolic flux investigation in the metabolism of *C. necator* cultivated using glycerol.

Keywords: Bioreactor cascade, Cybernetic models, Dynamic models, Elementary flux modes (EFM), Formal kinetic modelling, Hybrid models, Mathematical modelling, Metabolic models, Network modelling, Neural networks, Polyhydroxyalkanoates (PHA), Yield space analysis (YSA).

INTRODUCTION

Microorganisms constitute the most pristine and simple life form existing on Earth that can multiply (reproduce) themselves using their own biological apparatus. Due of their ostensible simplicity, microorganisms have acted as model organisms for biochemical, genetic and technological investigations. Among others, polyhydroxyalkanoate (PHA) producers (*i.e.*, various eubacterial genera and some archaea [1 - 5], genetically modified yeasts [6] and plants [7]) were investigated from different points of view: chemical (*i.e.*, composition of PHA homopolymers and copolymers /heteropolymers), metabolic (*e.g.*, biochemical pathways and regulation of biosynthesis and degradation), functional (*i.e.*, intracellular energy and carbon reservoirs [8] in diverse ecosystems [9]), and

technological (production starting from first and second generation renewable feedstocks, *i.e.*, sucrose-rich plants, grains, starch, molasses, lignocelluloses waste, whey, and biodiesel [4, 10, 11]) aspects were considered. Special attention was devoted to isolation procedures and processing technology in order to substitute well-established plastics originating from fossil resources, *i.e.*, poly(propylene), poly(ethylene), or other full carbon backbone polymers [5]. Depending on the type of polymers, raw materials, substrates, cultivation media composition, strains (wild type and genetically modified), cultivation techniques, downstream processing, and product recovery, an extensive number of experiments were performed by both the scientific community and the industrial sector in order to optimize PHA production in both economic terms and quality aspects.

Mathematical models are useful tools for optimizing microbial cultivation techniques, bioreactor performance and process kinetics. Such work encompasses the control of substrate(s) concentration, product(s) formation rate(s), measuring and regulatory systems, and, last but not least, the microbial metabolism, *i.e.*, the design of metabolic pathways, single cell- and whole population metabolisms [12 - 15]. As a well-known fact, biological populations and intracellular systems of cells are segregated in their nature. As a consequence, in the bioprocess engineering field, the complexity of cell reactions, structures and populations makes the creation of an appropriate structure of mathematical models very cumbersome. In addition, by linking the mathematical models with experimental data originated from metabolic, proteomic and genetic fields, one can disclose new features of bacterial physiology and sound and well-grounded interpretations of experimental results. Through intermittent crosschecking of models with experiments and *vice versa*, the improvement of knowledge can be significant, and the planning of subsequent, better targeted experiments, becomes possible [16, 17].

According to current model-theory, models can be classified into several classes and related counter-classes: descriptive/explanatory, deterministic/stochastic, discrete/continuous, verbal/non-verbal, unstructured/structured, black-/grey-/ white-box, non-mathematical/mathematical, and distributed (non-segregated/ segregated). Two or more of these classes can be combined and the resulting

CHAPTER 4

Bioreactor Design and Biochemical Consideration for PHB Production: Bioengineering Approaches

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Abstract: Considerable interests and widespread studies in the development of biodegradation of plastic materials have been carried out in order to overcome the environmental problems associated with petrochemical plastics waste. Among the various biodegradable polymers, poly(3-hydroxybutyrate) (PHB) is an attractive substitute for conventional petrochemical plastics due to similar properties to thermoplastics and elastomers, and complete biodegradability upon disposal under various environments. PHB is the most famous member of polyhydroxyalkanoates (PHAs) and can be accumulated as an intracellular carbon/energy source for various microorganisms. Synthesis of these distinct granules occurs when there is a growth limiting component in the presence of excess carbon source. The use of PHB in a wide range of applications has been hampered mainly by their high production cost compared with petrochemical based polymers. The fermentation performance, carbon

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substrate, the isolation of new microorganisms with high growth rate and potential of production as well as yield and recovery method affect the production cost of PHB. To overcome mentioned problems, knowing the specific strategies such as controlled systems is necessary. In controlled cultivation- and production systems like bioreactors, high cell density occurred. Hydrodynamic and mass transfer behaviors are important in gas liquid bioreactors. Oxygen transfer and residence time distribution must be monitored on-line. The mixing characteristics of gas liquid bioreactors are often intermediates between the characteristics of plug-flow and well mixed flow. This phenomenon is modeled using two methods: the axial dispersion model and tanks-in series model. In this chapter after a glance introducing of PHB and producing bacteria which grow on different carbon sources in batch, fed-batch, and continuous systems have been reviewed and compared to each other from the productivity point of view. Also, a special interest has been done on inhibition kinetics as well as modelling of gas and mass transfer in different bioreactors.

Keywords: Axial dispersion model, Bioreactor cascade, Bioreactors, Fed-batch cultivation, Fermentation, Forced-liquid bioreactors, Hydrodynamics, Mass transfer behavior, On-line monitoring, Polyhydroxyalkanoates (PHA), Poly(3-hydroxybutyrate) (PHB), Process design, Tanks in a series model.

INTRODUCTION

Pollution by plastics is one of the major concerns all over the world. The major factors that attract these synthetic polymers are that they are cheap can easily be produced from petrochemicals, and are very durable, flexible, and of low density. These synthetic plastics are inevitable for day-to-day human life. The main disadvantages of these polymers are their non-biodegradable characteristics and the fact that they are produced from non-renewable fossil resource. Hence, there is a need for alternative sources of plastics that can be produced from renewable resources which in addition should be biodegradable. Several researchers are constantly involved in the search for alternative sources of plastics; this has resulted in the discovery of biodegradable polymers in several life forms such as plants, microbes, and animals. Among these, microbes are the preferred source because the culture conditions during the respective bioprocesses can be easily controlled and monitored. Several microorganisms such as bacteria can synthesize polymers like poly(3-hydroxybutyrate) (PHB) that are biodegradable, and utilization of these biopolymers will help to reduce the dependency of industry on

petroleum derived plastics. Bacterial PHB can be produced from renewable resources, and offers ecological advantages if compared to thermoplastics and elastomers produced from fossil carbon sources [1].

PHB

PHB is a short chain length (*scl*) biopolymer with several properties similar to poly(propylene) (PE) and poly(propylene), and it can be easily degraded both aerobically to CO_2 and water or anaerobically to CO_2 and methane. PHB constitutes a rather crystalline material with high melting temperature and a high degree of crystallinity. The physical and mechanical properties of this material are similar to petrochemical thermoplasts, but PHB is stiff and brittle and the degree of brittleness depends on the degree of crystallinity, glass transition temperature, and microstructure. One of the main drawbacks of this polymer is that it is thermally unstable, which leads to decrease in viscosity and molar mass during processing [2].

PHB was the first PHA to be discovered and nowadays is the most widely studied and best characterized representative of the entire PHA family. It is accumulated as membrane-enclosed inclusion bodies in many bacteria and archaea at mass fractions up to 80% of the dry cell mass (CDM). It has mechanical properties very similar to conventional plastics like PP or PE and can be extruded, molded, spun into fibers, blown to films and used to generate polymer blends and composites. PHB and other PHA polyesters can be produced by various species of prokaryotic organisms [3 - 5]. Due to its biodegradability and other extraordinary properties, PHB could be ideal for use in medicine (*e.g.*, for hard and soft tissue implants, dressing and sewing material, tissue growth materials), pharmacy (controlled-release drugs), food packaging, or agriculture [6].

From a commercial point of view, PHB still lags behind the petroleum-based synthetic plastics. As the major drawback for a broader commercialization, the high production cost, which is dominated by approximately 40-50% by the raw material costs, has to be mentioned [7]. To attain bulk commercial viability and to further improve the sustainability profile of the entire PHB production chain, it is desirable to use waste and surplus material as feedstock for PHB biosynthesis. In

CHAPTER 5

Recovery and Characterization of Polyhydroxyalkanoates

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Abstract: Polyhydroxyalkanoate (PHA) production cost is dependent on several factors like substrate, chosen production strain, cultivation strategy and, to a still underestimated extent, on the downstream processing needed to recover PHA from microbial biomass. The availability of green and cheap technologies for PHA recovery is crucial for the development of a reliable and sustainable PHA production chain. Inexpensive and scalable recovery schemes need to be devised to achieve low-cost production that is competitive with traditional thermoplastics. Hence, in order to maximize both biomass growth and PHA productivity, one has to carefully optimize the operation conditions; this aspect of bioprocesses is of major economic importance in a bioprocess. PHA, a group of biobased microbial biopolyesters subjectable towards biodegradation constitute promising candidates to potentially substitute diverse conventional petrol-based plastics. As a drawback, PHA´s too high production cost still hampers their success on the on the market. The most prominent cost generating factors are the upstream processing, the bioprocess (fermentation), and, last, but not least, thus

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downstream processing dedicated to PHA recovery from microbial biomass. Downstream processing severely accounts to the economics of the overall process. A broad range of diverse recovery techniques can be found in the literature, most of them studied exclusively on small bench scale, with some exceptions which performed well also during industrial operation. Recovery by solvent extraction, chemical or enzymatic digestion of non-PHA cellular material, mechanical disintegration of cells, supercritical fluid extraction, flotation techniques, gamma irradiation and, more recently, the use of aqueous two-phase systems are reported. The present chapter summarizes investigated recovery methods and compares them in terms of efficacy and resulting product quality (*e.g.*, purity and impact on molar mass).

Keywords: Acetone, Aqueous two phase systems (ATPS), Characterization, Chemical digestion, Downstream processing, Enzymatic digestion, Mechanical disruption, PHA non-solvents, PHA recovery methods, Polyhydroxyalkanoates (PHA), Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) (PHBHV4HB), Purification, Solvent extraction, Supercritical fluids (*sc*F).

INTRODUCTION

PHA: Definition and Types

"Biodegradable polymers" are those polymers which are disintegrated by the enzymatic action of living organisms; they undergo complete degradation either in the presence of oxygen, in the absence of oxygen, or under both aerobic and anaerobic conditions. Aerobic degradation leads to the synthesis of CO_2, H_2O, and biomass, whereas degradation under anaerobic conditions generates hydrocarbons, methane, and biomass. Polyhydroxyalkanoates (PHAs) are clusters of biodegradable polymers which are accumulated intracellularly in various bacteria and archaea, usually at ample availability of exogenous carbon source, paralleled by restricted availability of nutritionally important factors like nitrogen, phosphate, oxygen, or minor nutrient compounds [1 - 3].

Based on the length of the carbon chain, PHA can be divided into three categories: Short chain-length PHAs (*scl*-PHA), consisting of C3–C5 monomeric building blocks; medium chain length PHAs (*mcl*-PHA), consisting of C6–C14 monomeric building blocks, and *scl-mcl* PHAs which contain C3-C14 monomers. Whereas *scl*-PHAs are classically rather crystalline, brittle thermoplastics,

mcl-PHA are known as more elastomeric latexes. *scl-mcl* PHAs display properties located between these two extremes, depending on the ratio of *scl*- and *mcl*-monomers in the copolyester.

Depending on their composition on the monomeric level, PHA can be divided into two categories [1 - 3]:

Homopolyesters: *E.g.*, poly(3-hydroxybutyrate) (PHB); PHB exclusively contains repeating units of (*R*)-3-hydroxybutyrate (3HB) and constitutes the best known representative of the entire PHA family; PHB is produced by numerous prokaryotic species. *Ralstonia eutropha* (today: *Cupriavidus necator*) is known to produce > 80% PHB of the dry cell mass (CDM) by using various carbon sources. Homopolymers generally exhibit good mechanical properties for the practical application.

Heteropolyesters (Copolyesters and Terpolyesters): Incorporation of different hydroxyalkanoates units into the PHB matrix results in PHA heteropolyesters with improved physical properties compared to the homopolymer. Heteropolyesters harboring building blocks with either side chains or backbones differing from 3HB are known as copolyesters, whereas heteropolyesters with at least three different building blocks with both side chains and backbones different from 3HB are termed terpolyesters. Many bacteria are known to synthesize copolyesters consisting of different hydroxyalkonoates of medium chain length (*mcl*-PHA) depending on their intrinsic PHA biosynthesis pathway and the applied carbon source.

A *scl*-copolyester consisting of 3HB and 3-hydroxyvalerate (3HV), namely PHBHV, has been investigated most extensively among the PHA copolymers and applied to manufacture commercial products. The addition of structurally related precursors, *e.g.*, propionic, valeric, or margaric acid as co-substrates usually allows for the production of PHBHV copolyesters. In addition, specialist production strains like *H. mediterranei* are reported to produce PHBHV from structurally unrelated carbon sources such as glucose or glycerol.

CHAPTER 6

Current and Emerging Advanced Analytical Technologies for Biopolyesters Characterization

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Abstract: Microbial polyester polyhydroxyalkanoate (PHA) is a carbon-neutral and environmental-friendly material with high commercial value due to its biodegradability and biocompatibility properties. PHA was originally thought to comprise of a single monomeric repeat unit of 3-hydroxybutyrate, but this notion has been since overthrown with the discovery of other chemically-distinct PHA monomers. To date, more than 150 PHA monomers have been documented. A PHA molecule may consist of two or more PHA monomers, endowing PHA polymer with high chemical diversity. This enables PHA materials of varied properties to be produced and tailored for application-specific purposes. However, the sheer number of chemically-diverse PHA monomers has also made the task of PHA analysis an extremely challenging one. Numerous techniques have been exploited for the detection, quantification, and characterization of microbial intracellular PHA and PHA polymers. New techniques are also continuously

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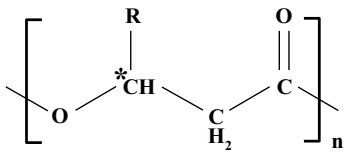
being developed with advancing instrumentation capabilities. This book chapter introduces the basic working principles underlying current and emerging PHA analytical techniques, and summarizes key protocols and information related to these techniques. The potential applications of emerging techniques are also highlighted and discussed.

Keywords: Biopolyester, Capillary electrophoresis, Characterization, Detection, Fluorescence spectroscopy, Fourier transform infrared spectroscopy (FTIR), Gas chromatography-flame ionization detection (GC-FID), Gas chromatography-mass spectroscopy (GC-MS), MALDI-TOF-MS, Mass spectroscopy (MS), NMR spectroscopy, PHA staining, Polyhydroxyalkanoates (PHA), Quantification, Techniques, Transmission electron microscopy (TEM), Wet scanning transmission electron microscopy (wet STEM).

INTRODUCTION

Polyhydroxyalkanoate (PHA) is a naturally-occurring biopolyester, which is synthesized and stored as intracellular granules in microbes. Since its discovery by Lemogine in 1926 [1], PHA has been recognized as a principal biopolyester synthesized by microbes [2]. At the molecular level, naturally-occurring PHA polymer is isotactic and comprises of exclusively chirally pure (*R*)-*β*-hydroxy fatty acids monomers [3], also termed as "PHA monomers". PHA monomer has a pendant *R* group, which is usually a saturated alkyl group that varies in carbon length from methyl (C_1) to pentadecyl (C_{15}) [4] (Fig. **1**); but also takes the form of unsaturated alkyl groups, branched alkyl groups, substituted alkyl groups although these forms are less common [5]. More than 150 different PHA monomers have been identified [6] and this number continues to increase with the discovery of new naturally-occurring PHA monomers [7], the chemical or physical modification of naturally-occurring PHA [8], and the creation of genetically-modified organisms (GMOs) to produce PHA with specific functional groups [9]. Depending on the total number of carbon atoms within a PHA monomer, PHA can be classified as either short-chain length PHA (*scl*-PHA; 3 to 5 carbon atoms), medium-chain-length PHA (*mcl*-PHA; 6 to 14 carbon atoms), or long-chain length PHA (*lcl*-PHA; 15 or more carbon atoms) [10]. A PHA molecule typically consists of 600 to 35,000 monomer units [10] and has a molecular mass between

50,000 and 1,000,000 Da [11].



Poly(3-hydroxyalkanoate)

R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Fig. (1). Chemical structure and nomenclature of PHA. The nonmenclature and carbon number for PHA compounds is determined by the functional alkyl *R* group. Asterisk denotes chiral center of PHA monomer. Adapted from [4], with permission.

There has been notable research and commercial interests in PHA due to its biodegradability [12 - 14] and biocompatibility [15 - 17]. Various combinations of chemically-distinct PHA monomers constitute to the chemical diversity of PHA, endowing PHA with a wide range of polymer properties [18], which may be tailored for specific downstream applications. A plethora of applications and potential applications have been identified for PHA and its monomers. These applications include biodegradable packaging materials, plastic coatings, pressure sensitive adhesives, biomedical products (tissue regeneration applications, scaffold for tissue engineering, surgical sutures, stents and controlled drug delivery matrices), and pharmaceutically-active compounds (fungicides, flavors, pheromones, vitamins, drugs, *etc.*) [11, 18 - 22]. Recently, there is a growing interest in *in vitro* PHA production *via* enzyme-catalyzed and metal-catalyzed reactions in order to expand the arsenal of PHA monomeric units, and thereby,

CHAPTER 7

3HB-Based Copolymers and Unusual PHA Homopolymers

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Abstract: Polyhydroxyalkanoates (PHAs) are bio-based polyesters synthesized by bacteria as an intracellular storage material. PHAs can be produced from renewable biomass without using fossil resources, and thus are environmentally friendly plastics. The most common PHA, poly[(*R*)-3-hydroxybutyrate] [PHB or P(3HB)], can be synthesized in large quantities by bacterial fermentation, but has rigid and brittle properties. Therefore, attempts have been made to improve its material properties. This chapter focuses on the recent progress in improving two types of PHA, 3HB-based copolymers and unusual PHA homopolymers, which show improved material properties and/or cannot be synthesized in nature. 3HB-based copolymers, that not only includes 3-hydroxyvalerate, 3-hydroxyhexanoate, and long chain 3-hydroxyalkanoate-containing copolymers, but also 3-hydroxy-4-methylvalerate, 3-hydroxy-3-phenylpropionate, 3-hydroxy-2-methylbutyrate, and lactate-containing copolymers have been reviewed. Additionally, ultrahigh-molecular-weight PHB and medium-chain-length PHA homopolymers are highlighted as unusual homopolymers. These polymers have notable characteristics and are expected to expand the range of PHA applications.

Keywords: Aromatic building blocks, Branched building blocks, Copolymer, Copolymerization, Homopolymer, Metabolic pathway, Molecular weight, Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) P(3HB-*co*-3HHx), Ultrahigh-molecular weight.

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INTRODUCTION

Many bacteria and archaea synthesize a polyester known as polyhydroxyalkanoate (PHA) from biomass such as sugars and plant oils. Similar to fat in humans, bacteria use PHA as energy storage material that can be degraded under starvation conditions to generate energy. As a macromolecular material, PHA has superior properties such as thermoplasticity and biodegradability; because PHA is an environmentally friendly and renewable material (Fig. **1**), it can be widely used as an alternative to petroleum-based plastic.

However, in order to commercialize PHA, low production costs, low environmental burden throughout the life cycle, and practical material properties are required. In this chapter, we focus on the recent progress in PHA research in the past decades and overview 3-hydroxybutyrate (3HB)-based copolymers and unusual PHA homopolymers cannot be naturally synthesized.

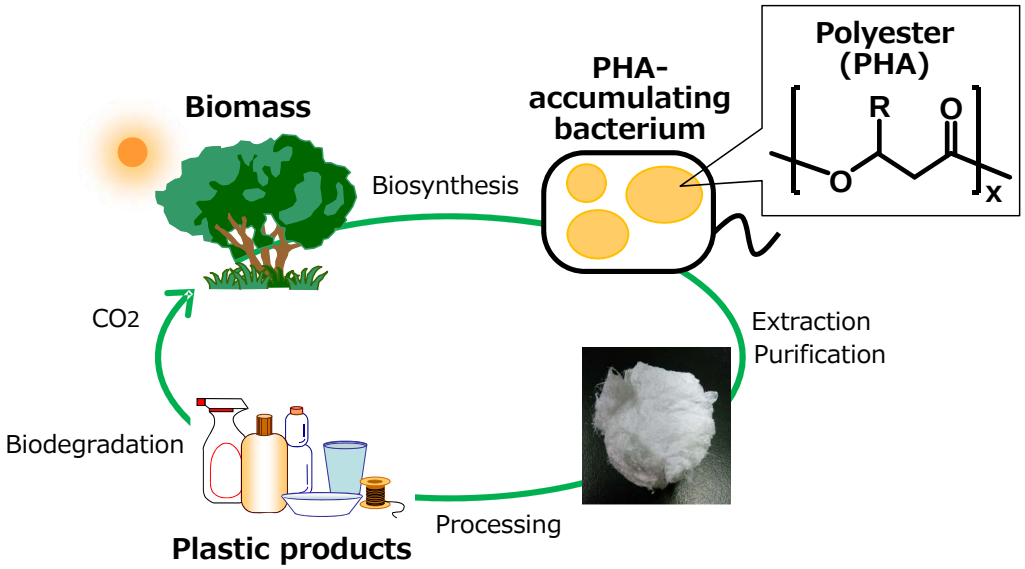

Fig. (1). Polyhydroxyalkanoate (PHA) and global carbon cycle.

OVERVIEW OF PHB AND PHBHV

The bacterial polyester polyhydroxyalkanoate (PHA) was first discovered in 1926. A research team at the Pasteur Institute in France found that PHA constituted the intracellular material of *Bacillus megaterium* (*B. megaterium*) [1]. The PHA

identified was poly[(*R*)-3-hydroxybutyrate] [PHB or P(3HB)], which is the most common PHA. Ninety years since its discovery, hundreds of natural PHA-producing bacteria and at least 150 PHA monomers have been reported [2]. In this section, PHB and P[3HB-*co*-(*R*)-3-hydroxyvalerate] [PHBHV or P(3HB-*co*-3HV)] copolymers, which have been widely studied and are well-characterized, are reviewed in the context of their biosynthesis and material properties.

PHB is produced by a wide range of bacteria and some archaea. The bacterium *Ralstonia eutropha* (*R. eutropha*) is the best-known natural producer of PHA; in this bacterium, the polymer is synthesized from acetyl-coenzyme A (acetyl-CoA) in reactions catalyzed by three enzymes: 3-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC). *R. eutropha* can accumulate large amounts of PHB (80% of the cell dry mass) from various biomass resources such as sugars, organic acids, and plant oils and carbon dioxide during autotrophic growth. Given its hydrophobic properties, PHB aggregates in cells and is accumulated as intracellular granules (Fig. **1**). Through genetic engineering, non-PHA producers such as *Escherichia coli* (*E. coli*) [3] and tobacco [4] have been employed as hosts for PHB production.

PHB has functions such as thermoplasticity, biodegradability, and biocompatibility. The thermal properties of PHB are similar to those of polypropylene, which is used in packaging, labeling, and textiles (Table **1**). However, the mechanical properties of PHB exhibit stiff and brittle features because of its high crystallinity. As shown in Table **1**, elongation to break of PHB is only 5%, indicating poor elasticity. To improve the physical properties of PHB, copolymerization with the 3HV unit was investigated.

Table 1. Properties of PHAs and chemosynthesized polymers.

	T_m [a] [°C]	T_g [b] [°C]	Young's Modulus [MPa]	Tensile Strength [MPa]	Elongation to Break [%]	Ref.
PHB	177	4	3500	43	5	[102]
Ultrahigh-molecular-weight PHB	185	4	-	400	35	[102]
P(3HB-*co*-20 mol-%-3HV)	145	-1	800	20	50	[102]
P(3HB-*co*-10 mol-%-3HHx)	127	-1	-	21	400	[102]

CHAPTER 8

Microbial Bio-polyesters: Crystallinity and Mechanical Properties

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Abstract: Crystalline structure and morphology of the solid state of thermoplastic semi-crystalline polymers strongly affect their properties and performances. This is particularly evident on mechanical properties of poly[(*R*)-3-hydroxybutyrate] (PHB), and its copolymers, blends, composites, and nanocomposites. The organization and the morphology of the crystals and the interconnection between the crystalline and the amorphous regions can influence and govern the mechanical properties of PHB based materials.

The presence of a rigid amorphous fraction, which produces a stiffening of the amorphous segments at the crystal/amorphous interface, can contribute to the progressive change of the mechanical properties in PHB based materials.

Properties of PHB based materials may be tuned and controlled by blending with other polymers, by addition of properly selected plasticizers, nucleating agents, as well as by addition of fillers and nanofillers.

In the present Chapter the influence of the morphology and the crystalline content on the mechanical properties of PHB based materials is analyzed and discussed.

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Keywords: Blends, Composites, Crystallinity, Mechanical properties, Mobile amorphous fraction, Morphology, Polyhydroxyalkanoates (PHA), Poly[(R)-3-hydroxybutyrate] (PHB), Polymer processing, Polymers, Rigid amorphous fraction.

INTRODUCTION

It is well known that the physical properties of a thermoplastic semi-crystalline polymer, as for example thermal, mechanical and gas permeability properties, are markedly influenced by the crystalline structure and morphology. Generally, these characteristics are controlled by the solidification and processing conditions.

A full knowledge of the structure and morphology of the crystalline phase and its quantification are crucial steps for both the comprehension and the prevision of the final properties of a material, as small changes in crystallinity can dramatically modify its properties. The principles that govern the relationships between structure and properties of the biopolymers are the same that determine the behavior of fuel-derived polymers.

Biopolymers are very encouraging sustainable alternatives to conventional petrochemical-derived products [1 - 4]. Nevertheless, their extensive use has been hindered until now by some insufficient properties relevant for practical applications, which make these polymers not equivalent to conventional thermoplastics. Many attempts to improve their properties have been done, mainly based on preparing mixtures with various different compounds or with other polymers [5].

In this chapter, an analysis of the mechanical properties, especially related to the crystalline degree, of poly[(*R*)-3-hydroxybutyrate], some of its copolymers, blends, composites, and nanocomposites is presented and discussed.

Poly[(*R*)-3-hydroxybutyrate] (PHB)

The homopolymer poly[(*R*)-3-hydroxybutyrate], which we will further report as PHB, is the most widespread, and best characterized member of the polyhydroxyalkanoate (PHA) family. PHAs are a group of biocompatible and completely biodegradable polyesters, synthesized by many bacteria as intracellular carbon and energy reservoir [6]. Owing to their biodegradability, they

can be utilized to make a variety of products, in particular for application in single-use packaging and agriculture.

PHB is a semi-crystalline isotactic stereo-regular polymer with 100% (*R*)-configuration. It is hydrophobic, water-insoluble, inert, biocompatible and indefinitely stable in air. The mass-average molar mass of PHB is usually in the range $1 \cdot 10^4$-$1 \cdot 10^6$ g mol^{-1}, with a polydispersity of about 2 [6].

The as-received isolated from bacteria PHB is a highly crystalline powder, with average crystallinity of approximately 65% [7 - 9], whereas the *in vivo* PHB is an amorphous polymer [10, 11].

Upon solidification from the melt, PHB adopts a spherulitic morphology. Owing to its biosynthetic origin, PHB is free from any residual contaminating catalyst, which leads to a low nucleation density and large dimensions of the spherulites [12]. When observed by optical microscopy, PHB spherulites typically show alternating extinction bands, due to the twisting of the lamellae. Band spacing increase with the increase in crystallization temperature has been reported [13]. The overall crystallization of PHB shows a maximum rate around 90 °C [14].

The bacterial PHB can be crystallized in two modifications: the α- and the β-forms [15]. The most common crystal structure of PHB is the α-form, which is produced under the typical conditions of melt, cold or solution crystallization. The α-form is characterized by two antiparallel chains in the left-handed 2_1 helical conformation packed in an orthorhombic unit cell having axes a = 0.576 nm, b = 1.320 nm, and c (fiber axis) = 0.596 nm [16]. Conformational analysis based on intramolecular potential energy calculation indicated that the ester groups of the antiparallel chains within the unit cell of the PHB α-form are nearly at the same level, and that the angle between the dipoles is ~60°, suggesting that the dipole–dipole interaction is the main factor governing the molecular packing [17].

The thermal properties of PHB are close to those of polypropylene, PP, as detailed in (Table **1**), in which the average mechanical properties of PHB, PP and low-density- and high-density- polyethylene, LDPE and HDPE, are reported for comparison. Besides the glass transition temperature and the melting temperature, also the Young's modulus and the tensile strength of PHB are similar to those of

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> This second volume in the sequel of Microbial Polyester covers all important aspects of polyhydroxyalknaote production in bioreactors including kinetic models, their material properties and modification by blending and formation of composites and finally their evaluation of the sustainability. This book gives a spotlight on the exciting field of biodegradable plastics and is recommended to both newcomers and experts interested in research and application of polyhydroxyalkanoates.

Manfred Zinn, Prof. in Biotechnology at HES-SO // Valais - Wallis

Martin Koller

After completing his doctoral thesis under supervision of Prof. Gerhart Braunegg, one of the most eminent pioneers in polyhydroxyalkanoate (PHA) research, Martin Koller was awarded his PhD degree in 2005 from Graz University of Technology, Austria for his work on PHA production from dairy surplus streams; these activities were part of research in the EU-FP5 granted project WHEYPOL ("Dairy industry waste as source for sustainable polymeric material production"). Afterwards, he became a highly experienced senior researcher in the field of bio-mediated PHA production, encompassing design and development of continuous and discontinuous fermentation processes, and novel downstream processing techniques for sustainable biopolymer recovery from microbial biomass.

His focus of research is enhanced cost-efficiency of PHA production from surplus materials using both eubacteria and halophile archaea as whole cell biocatalysts. He holds numerous research articles in high ranked scientific journals, authored several chapters in biotechnological scientific books, gave a broad number of invited and plenary lectures at international conferences, and is active member of the Editorial Boards of distinguished scientific journals. From 2010 to 2012, he acted as coordinator of the EU-FP7 granted project ANIMPOL ("Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products") which investigated the value-added conversion of waste streams from the animal processing industry towards structurally diversified PHA biopolyesters and their marketable follow up products.

In addition to biopolymer exploration, he was also active in microalgal research and has long-year experience in biotechnological production of marketable compounds like bioethanol, polysaccharides, lactic acid or polyunsaturated fatty acids from renewable resources by various microorganisms such as yeasts, chlorophyta, bacteria, archaea, fungi or lactobacilli.