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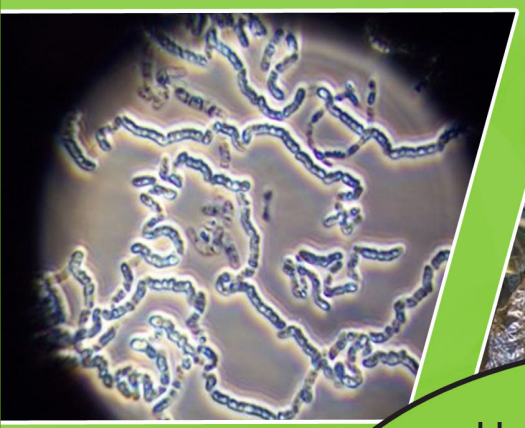
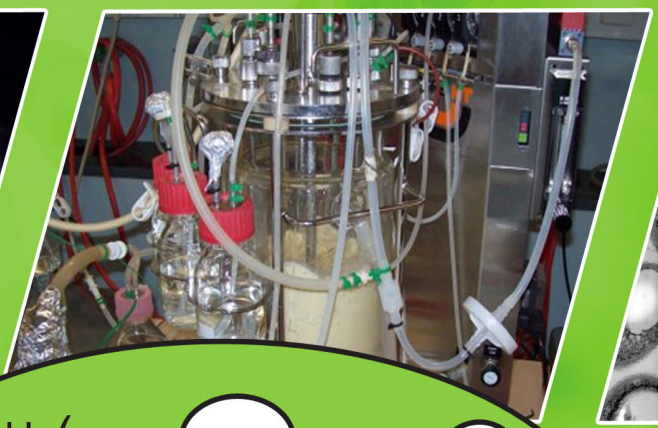
Recent Advances in Biotechnology

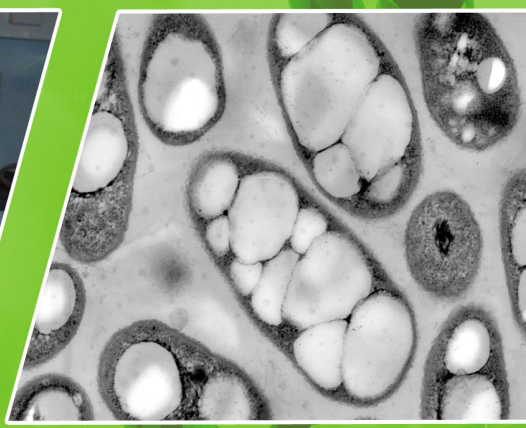
Volume 1

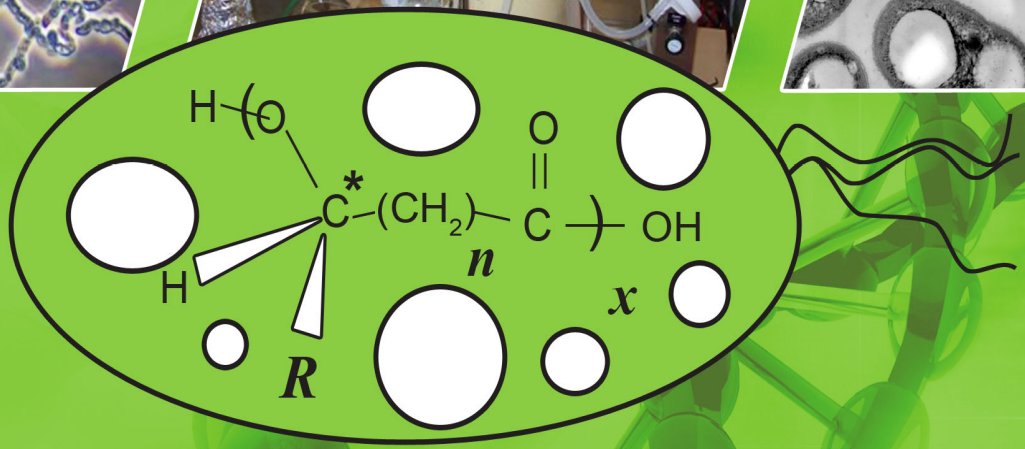
Microbial Biopolyester Production, Performance and Processing

Microbiology, Feedstocks, and Metabolism









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(Volume 1)

Microbial Biopolyester Production, Performance and Processing

Microbiology, Feedstocks, and Metabolism

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FOREWORD

I am very happy to receive an invitation from Dr. Martin Koller to write something on my motivation for PHA research, how the knowledge about PHA and PHA-related R&D has changed in the time since I started with this topic, and what my ideas are for the future of PHA. I take this invitation as an initiative to think the past and future of these fascinating materials.

I started my PhD thesis in 1986 under the guidance of Professor Robert M Lafferty at the Graz University of Technology (TU Graz), Austria. At that time, as an organic chemist working on chemical synthesis of Nylon-6 under explosive or flammable conditions in organic solvents, I was very much attracted by the idea of using gentle conditions like aqueous phase, room temperature and normal pressure to make plastics. My initial research was on fermentation process development using *Alcaligenes latus* DSM 1122, 1123 and 1124 for production of copolymers PHBHV consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). At that time, ICI successfully launched the PHBHV product trademarked as BIOPOL, the whole world was enthusiastic for bioplastics as they seemed to solve the environmental and petroleum resource crisis. At the Institute of Biotechnology (today: Institute of Biotechnology and Biochemical Engineering) of TU Graz, I was able to interact not only with my supervisor Professor Lafferty but also with Dr. Gerhart Braunegg, Dr. Walter Steiner and Dr. Helmut Schwab as well as many highly talented PhD students. Dr. Gerhart Braunegg is the inventor of the gas chromatography (GC) method for qualitative and quantitative PHA determination. Although this GC method was invented and established in the 1970s, it is still the most popular method for PHA studies, as manifested by its numerous citations in the recent literature. For that all PHA researchers should pay respects to Gerhart. In addition, Gerhart has done some excellent works on using waste substrates for PHA production. He also actively promotes the concept of continuous fermentation for PHA production. This concept has an in-depth impact on my research career, which I will describe shortly.

I left Graz in 1990 to conduct my postdoc studies in University of Nottingham in UK. My postdoc study aimed to use PHA as a matrix to encapsulate drug for sustainable release. Very soon we found that PHA *in vivo* degradation was too slow to meet the sustainable release goal compared with other polymers like polylactide (PLA), poly(lactide-*co*-glycolide) (PLGA) and human serum albumin. However, the flexible mechanical property combined with biodegradability and biocompatibility makes PHA a suitable material for implant tissue engineering applications, and this forms part of my career interest in my research conducted at Tsinghua University/Beijing where I become an independent principle investigator.

PHA research has been changed dramatically since I started my PhD thesis 30 years ago. The changes can be summarized in the following areas:

1. Much wider PHA diversity which evolves into "PHAome" similar to "Genome" or "Proteome";
2. Much more "Synthetic Biology" approaches for engineering PHA production strains in a more effective way;
3. Open (unsterile) and continuous fermentation processes for high cell density and high PHA contents growth;
4. High-value applications of PHA in areas of medical implant applications and smart material developments.

Since the breakthrough in weakening beta-oxidation in *Pseudomonas* spp. by our lab in 2010, it has become possible to precisely control the structures of PHA either in the form of homopolymers, random copolymers or block copolymers. This will really achieve tailor-made PHA based on property requirements. Since *Pseudomonas* spp. are the strains used to generate the "PHAome", the economy to obtain diverse PHA is very important depending on the ability to reach high cell density growth for *Pseudomonas* spp. In this area, Dr. Bruce A Ramsay has done some excellent researches to realize high cell density growth of *Pseudomonas putida* containing over 50% PHA. The contribution of Bruce also contains the efficient extraction of PHA from *Pseudomonas* spp. In the future, PHAome produced by high cell density growth of *Pseudomonas* spp. will allow diverse PHA be produced in economically competitive way.

With the rapid developments of synthetic biology, many possibilities can be exploited to improve PHA production strains, including promoter engineering and ribosome-binding-site (RBS) optimization to enhance PHA content, minimized genome to, *inter alia*, increase substrate to PHA conversion efficiency, reprogramming cell growth pattern to accelerate cell growth, new pathway assembly for PHAome from glucose only, mixed substrate fermentation for single PHA molecules, morphology engineering the bacterial cell shapes for enhanced PHA accumulation and reduction on downstream purification cost. All of those efforts should lead to the significant reduction on PHA production costs.

Since PHA production cannot compete with the petrochemical plastic industries as it suffers from high consumptions on energy and freshwater, discontinuous processing, low product concentration and thus high product recovery cost as well as low substrate to product conversion efficiency. Therefore, to make PHA production competitive, we need to develop fermentation technology which is energy and fresh water saving and run in a continuous way instead of a batch way. To meet this need, a seawater technology based on *Halomonas* spp.

grown in seawater in an unsterile and continuous process has been developed. Since seawater contains around 35 g/L NaCl and *Halomonas* spp. prefer higher pH, the process allows *Halomonas* spp. to grow in an open and continuous way for at least two months infection free. This seawater biotechnology achieves fresh water saving, energy saving and reduces process complexity combined with use of low cost fermentors, it significantly reduces the production cost of PHA and possibly other bioproducts. The *Halomonas* spp. have been engineered to allow them to become a platform for low cost production of biofuels, biomaterials and chemicals.

This vast diversity constituting the "PHAome" is waiting for exploitation. Except for very few PHAs that are commercially available from the market for application developments, such as PHB, PHBHV, P3HB4HB and PHBHHx, all other PHAs are produced by individual labs across the world in very small amounts for academic curiosity. Moving advanced materials like PHAs from the laboratory to the marketplace to address critical challenges in environment, energy, transportation, healthcare, and other areas of application concerns takes far too long, and at too high a cost. How to accelerate the pace of discovery and deployment of advanced PHA materials has been a central question for all researchers and stakeholders in this field. All these depend on the availability of the diverse PHAs in sufficient quantities and with consistent properties for studying their thermal and mechanical properties as well as other application potentials. It should be a global effort to establish platforms to supply diverse PHA based on PHAome information in sufficient quantities and material property consistency for various application developments. The understanding of the PHAome concept should allow us to construct a suitable bacterial platform that is able to provide consistent PHA molecular structures with less molecular weight variations for constant material properties. This is very important for commercial developments.

I hope this information is helpful for PHA researchers.

Thank you for your attention!

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PREFACE

This *e*Book volume sheds light on contemporary R&D endeavors managed in various research institutions all over the world; these endeavors have one aim in common: *Replacing established plastics usually stemming from the conversion of fossil feed stocks by "bio-inspired" alternatives provided by nature!* The utmost significance of these endeavors shall be visualized by the annual pile of plastics of about 330 Mt produced worldwide; the predominant part of these plastics is highly resistant against (bio-)degradation, and only an evanescent share of about 20% of spent plastics is subjected to recycling procedures. Sustainable alternatives, so called "environmentally degradable plastics", exist, but do not even cover 5% of the global plastic market. In addition, although very common today, the term "environmentally degradable plastic" is used highly ambiguously and often explicitly incorrectly; in many cases, so called "bio-plastics" do not conform to the attributes "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 versatility, 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.

My personal motivation to edit this *e*Book volume started already more than one decade ago, when I was doing my Master thesis on novel, ecologically benign methods for extraction of PHA from microbial biomass in the group of Professor Gerhart Braunegg at Graz University of Technology. Since then, I got more and more fascinated by these unique biopolyesters. Not only the multiple and complex metabolic roles, the multifaceted functions of PHA in divers 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. After this project, several other research groups worldwide investigated whey-based production of PHA and other valued bio-products; this gave reason for a chapter dedicated to this topic in the volume at hand (see Chapter 2). 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 gratitude 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 the two volumes of this *e*Book issue, all single steps, starting from the intracellular procedures on the genetic and enzymatic level, the selection of the adequate raw materials, the biotechnological production process including kinetic analysis and mathematical modelling, process engineering aspects regarding fermentation modes, feeding regimes and bioreactor selection, until the downstream processing for product recovery and refining have to be understood and optimized; this goes in parallel with market issues and holistic consideration of sustainability aspects. Fig. (**1**) illustrates the seven pillars which have to be addressed to come to efficient PHA production processes. The volume at hand concentrates on microbiological, genetic, enzymatic, substrate-related and metabolic issues, whereas aspects of engineering (fermentation technology and downstream processing), kinetic modelling, and polymer characterization, as well as sustainability- and market issues will be the focus of the forthcoming volume 2.

THE CONTRIBUTING SCIENTISTS

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*" address this shortcoming in 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.

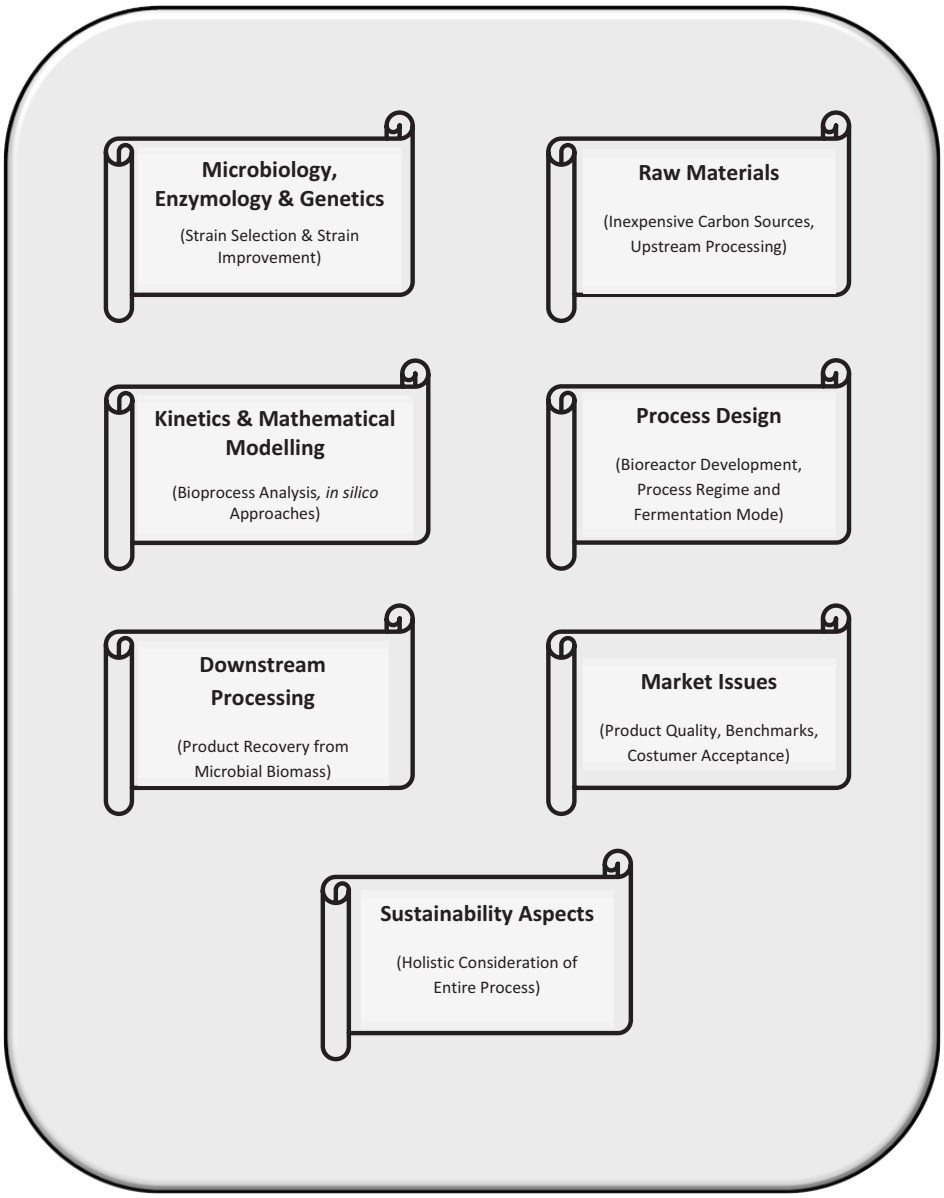


Fig. (1). The seven pillars of efficient PHA bioproduction.

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, I am convinced that these two volumes are outstanding composites of comprehensive articles covering all single aspects which are nowadays recognized to be decisive for sustainable and efficient production of microbial polyhydroxyalkanoate (PHA) biopolyesters. In the volume 1 at hand, major attention is devoted to microbiological aspects to search for new powerful PHA production strains, and to implement mixed microbial cultures to combine removal of ingredients from waste water bodies with PHA bioproduction; further a special chapter is dedicated to photoautotroph microbes which attract increasing interest based on their high potential for solar-driven PHA biosynthesis from CO_2. Genetic engineering as a tool to enhance the metabolic performance of PHA producing organisms is covered by another chapter. Due to the high importance of the carbonaceous raw materials needed as microbial feedstock for PHA production, several chapters provide either generic or specialized essays on this topic. The huge variety of different types of PHAs produced by various microbial strains allows for a vast range of different material properties, dependent on PHA´s composition on their monomeric level. This gave reason for different groups of authors to dedicate special chapters to well-described or novel PHA homopolyesters, and to the emerging field of medium chain length PHA (*mcl*-PHA), a fascinating new class of "biolatexes" and "bioelastomers".

The volume at hands, *"Part 1: The Microbe´s Point of View (Aspects of Microbiology, Feedstocks, and Metabolism)"* encompasses eight chapters dealing with aspects of microbiology (selection of powerful archaeal and eubacterial production strains, genetic engineering as a tool for optimized PHA production), and puts emphasize on the raw material selection by describing the potential of applying inexpensive carbon feedstocks for PHA bioproduction. The subsequent paragraphs shall give the respected readership an impression about the contents of the specialized chapter articles of this issue volume:

CHAPTER 1

Stanislav Obruca and associates from Ivana Marova´s group at the Technical University of Brno, Czech Republic, address the fact that the main obstacle until now preventing PHAs from finally succeeding on the plastic market is the still too high production cost, important portion of which is attributed to carbon substrate. These authors provide an extensive and up-to-date compilation of the incredible variety of inexpensive substrates which were globally investigated for their potential upgrading towards feedstocks for sustainable production of PHA. Hence, this chapter summarizes advances in biotechnological production of PHAs from various carbon-rich waste products stemming from different fields of industry, especially food industry and agriculture. Special attention is devoted to PHA production from inexpensive lipids, side streams from dairy-, sugar-, and biodiesel production, lignocellulosics, starch-based materials, diverse volatile organic acids, and exotic, precarious substrates such as

petrochemical plastic waste. The authors underline that substrates for PHAs production should meet several basic criteria such as availability in constant amount and quality during whole year, stability, low cost of collection, transportation and processing, and emphasize that, for ethical reasons, application of raw materials for biopolymer production must be in no conflict with human food chain.

CHAPTER 2

The volumes of generated surplus whey almost equal to those of the whole milk processed for cheese production, and by far surmount the needs of its restricted classical markets such as animal fodder, pharmaceutical matrices, or additives in food production. As a consequence, enormous quantities of whey are contemporarily disposed in aquatic environments, provoking prevailing ecological concern due to its considerable biochemical oxygen demand, and high expenses for the disposal. This was the motivation for our article describing efforts in the past and nowadays to apply whey as a substrate for biotechnological PHA production. We show that, using different production scales, production strains and production modes, surplus whey can be upgraded to the role of a carbon feedstock for the production of PHB homopolyester or, by switching to advanced feeding strategies and application of powerful novel production strains, to tailor-made PHA co- and terpolyesters with customer-oriented material properties. Microbiological, engineering, and economic aspects of whey-based PHA production by meso- and extremophile wild-type or genetically engineered prokarytotic production strains are covered. The route needed to make efforts in this field competitive with current PHA-production on prized feedstocks of nutritional significance is presented. This competitiveness is addressed both in environmental and economic aspects. In addition, the chapter addresses also alternative routes for sustainable and value-added conversion of surplus whey, *e.g.*, for generation of green energy carriers or various organic bulk- and fine chemicals.

CHAPTER 3

Gabor Rakhely and his colleagues from the Hungarian Academy of Sciences compiled a comprehensive review on the production of biopolyesters and related valued products from photoautotrophic microorganisms such as different purple non-sulfur-, sulfur photosynthetic- and cyanobacteria. The authors address the metabolic functions and particularities of anabolism and catabolism of these biomaterials in photoautotrophs and clearly visualize that these intracellular ongoing differ significantly from observations in heterotrophic PHA producers. Moreover, the authors present photoautotrophic PHA production by cyanobacteria and related organisms from simple, abundant carbon sources like CO_2 or syngas as one of the most promising routes to enable efficient PHA production, and discuss the impact of addition of organic substrates to improve the PHA yield. With this chapter, the authors clearly show that application of phototrophic microbes definitely can be regarded as a serious strategy for

efficient production of PHA homo- and heteropolyesters. PHA composition- and yield might be varied by the growth conditions and especially the illumination regime. Although at the moment the efficacy of phototrophic PHA production processes is significantly lower than for established technologies applying chemoheterotrophic microbes, it might be further improved by genetically modified strains and/or bioprocess optimization. Alternatively, waste utilization combined with CO_2 fixation might also constitute a viable approach.

CHAPTER 4

PHA production by mixed microbial cultures (MMC) as an alternative to application of pure microbial cultures became the central point of many research works. This motivated Luisa Serafim and colleagues from Portugal, to contribute the first comprehensive review on this topic. Firstly, they discovered wastewater treatment systems, namely those related with biological phosphorus removal, application of MMC became a process to be considered for polymer production intended to replace petrochemical derived plastics. The ability to store PHA provides microbial communities a competitive advantage for survival under transient conditions typical of waste treatment systems. By this way, MMC can continuously adapt to the operational conditions, increasing the share of PHA-storing organisms in the microbial community. As further benefit, this approach is connected to minimum requirements of sterility. MMC can produce PHA using organic waste or industrial by-products as substrates, allowing for their valorization. The use of waste, together with the lower requirements in sterility and process control, could signify a decrease on PHA production costs. This chapter intends to contextualize the use of MMC for PHA production, by describing the different approaches and comparing them with the traditional processes employing pure or genetically modified organisms. As a resume, the authors conclude that, although being challenging, PHA production by MMC constitutes a way of waste treatment through its valorization. Being PHA production a competitive advantage for microbial cultures, the enrichment in PHA-storing bacteria depends more on the operational conditions imposed to the process rather than the existence of sterility. PHA producing processes using MMC and waste as substrates could help to reduce the polymer costs but at the same time using the existing facilities of waste/wastewater treatment systems and residues on location could contribute to the overall profit of industries and waste water treatment plants.

CHAPTER 5

Christopher Brigham, Abdulrahman A. Kehail, and Jacob D. Palmer from the University of Massachusetts, USA, address the high versatility of the best investigated PHA producing microbe: *Ralstonia eutropha* (a.k.a., *Cupriavidus necator*). The authors point out two principal reasons for the popularity of *R. eutropha* for biotechnology: 1) the organism´s versatile metabolism and acceptance of a wide variety of carbon sources from sugars and

lipids to carbon dioxide and aromatic compounds for growth and bioproductions, 2) its capability to store a large amount of carbon as intracellular polymer. The article further underlines that this strain is not only known as the model system for PHA biosynthesis and mobilization, but, moreover, *R. eutropha* is being used as a host organism for heterologous bioconversions, such as the synthesis of "Eutrophanol" as a novel biofuel. This chapter chronicles the key discoveries of *R. eutropha* biology and presents the biomanufacturing context that inspired many research groups to tailor the organism as a novel biocatalyst. The authors emphasize that there are still many challenges to be faced and to be overcome to develop bioprocesses with *R. eutropha* as the biocatalyst. Firstly, the costs of PHA production must be lowered significantly to compete with the cost of petroleum-based plastic as raw material. Secondly, to engineer heterologous biosynthesis pathways in *R. eutropha*, the path forward to high yield productions must be clear.

CHAPTER 6

Another fascinating PHA producer is described by the contribution of Hua Xiang from the Chinese Academy of Science: *Haloferax mediterranei*, an extremely halophilic archaeon has shown promise for the production of the copolyester poly(3-hydroxybutyrate-*co*--hydroxyvalerate) (PHBHV) from many low-cost carbon sources of industrial origin, and can be considered as one of the rising stars on the sky of biotechnology. This extremophile organism, originally isolated from Mediterranean brines, can be cultivated in highly saline cultivation media at almost zero sterility precautions and produces high-quality PHBHV copolyesters from unrelated carbon sources which, after biomass harvest, can easily be recovered from the bioactive cell material. To obtain a complete understanding of PHBHV biosynthesis and to provide novel strategies for PHA production by haloarchaea, the complete genome of *H. mediterranei* has been deciphered, and a genome-wide investigation of the genetics and metabolism of this ancient haloarchaeon involved in PHBHV biosynthesis was performed only recently in the past few years. This exploration covers the identification of key genes for PHBHV biosynthesis, the specific precursor supplying pathways, the PHA granule modulation, and PHBHV mobilization, as well as its linkage to haloarchaeal-specific acetyl-CoA and propionyl-CoA assimilation. These advances have not only revealed many haloarchaeal-specific enzymes and pathways for PHBHV biosynthesis, such as the archaeal-type PHA synthases, the haloarchaeal-type β-ketothiolases with two distinct subunits, and the novel 3-hydroxypropionate pathway coupling CO_2 fixation into PHBHV, but have also provided strategies for bioengineering haloarchaea for economical production of PHBHV or for tailor-made PHAs of even higher value. In addition to PHBHV production, the chapter also reveals other valued compounds produced by this strain, such as carotenoid pigments, hydrolytic enzymes, xanthan-like polysaccharides, or antimicrobial halocins.

CHAPTER 7

Sergio Casella, Lorenzo Favaro, and Marina Basaglia from the University of Padova, Italy, emphasize that several phases of the PHA production chain can be identified where genetic tools can efficiently be implemented in view of possible industrial applications, starting from the microbial strain isolation and characterization, through all the biotechnological steps, until the downstream processing. Leaving aside the huge number of genetic modifications accomplished on many bacterial species to clarify basic biochemical, genetic and metabolic aspects of PHA metabolism, the chapter reveals that several intriguing attempts have been reported aiming at improving the performance of the microorganisms to be used for a possible PHA production process. Most of natural microorganisms can synthesize PHAs with varied monomers only at lab scale from structurally-related precursors, which are frequently expensive, not miscible in water, toxic even at low concentrations and high oxygen demanding. That is why researchers have addressed their attention to inexpensive and renewable unrelated carbon sources. Moreover, the exploitation of the metabolic potential of bacteria for the production of PHAs with tailor-made monomer composition from unrelated carbon sources rapidly became one of the main objectives. At the same time, the improvement of yield has to be faced not only with the modification of growth conditions, but possibly by re-engineering metabolic flux to drive carbon utilization toward PHA biosynthesis pathway.Most of the available information deals with the use of genetic tools for (*i*) characterization and identification of new isolates, (*ii*) testing new isolates for the presence of relevant genes involved in PHA production/degradation, (*iii*) metagenomic approach in complex environments for searching relevant genes (and strains), (*iv*) assessment of PHA genes expression and control of depolymerase genes, (*v*) metabolic engineering of producing and not producing bacterial strains, (*vi*) improving downstream processes, (*vii*) making PHA producing strains able to utilize different inexpensive carbon sources, (*viii*) cloning PHA genes in other recipient bacteria. The authors conclude that a wide range of bacteria capable of naturally producing PHA or those obtained through recombinant DNA techniques are not limited to those already described, but that many other organisms with desirable properties could be exploited in the future for producing recombinant PHAs.

CHAPTER 8

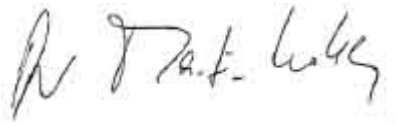
Bruce Ramsay contributes a chapter on a special class of PHA biopolyesters which attracts increasing attention due to numerous anticipated applications, namely on *mc*l-PHA. Accumulated only by a manageable group of related Pseudomonas species, these special polyesters are remarkable in their physic-chemical properties (especially low crystallinity and low glass transition temperature), structural diversity, chemical functionality, and areas of applications; the reader will learn that investigation of *mc*l-PHA properties and exploration of

special fields of their application are still in their infancy. *Mcl*-PHA is highly water resistant, yet readily susceptible to enzymatic degradation. They can, *inter alia*, be used in packaging, "biolatex", paints, toners, "smart adhesives" and in many biomedical applications but have yet to be produced commercially. *Mcl*-PHAs are most efficiently produced from carboxylic acids but can also be produced from many non-related substrates. Despite their numerous potential applications, *mcl*-PHAs cannot yet compete with the polymers currently used due to their high cost of production. The author describes recent advances in the biotechnological production and downstream processing for product recovery, as well as strain development in order to improve polymer quality and finally to reduce PHA production cost by increasing productivity, which should soon lead to the adoption of *mcl*-PHAs by industry. The author also emphasizes the need to implement new, unprecedented engineering strategies to accelerate the market break-through of *mcl*-PHA, such as multistage continuous approaches to tailor product composition, or optimized oxygen transfer by pressurized bioreactor facilities in order to generate high cell densities which increases the overall volumetric productivity.

With this issue volume, we wish to address scientists active in the field of biopolymer science, microbiologists, genetic engineers, and biotechnologists in general. 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, and can give you some insight into the fascinating world of these multifaceted biopolymers! In addition, we strongly believe that the issue volume at hands also attracts the attention of representatives of polymer industry, agroindustry, and biofuel industry. Do you have a carbonaceous waste stream to be upgraded to a feedstock for PHA production? Do you want to get to feet on the ground of innovative polymeric products? Is it conceivable to construe your production lines as a "biorefinery" system, where material stream now undergoing cost-intensive disposal could act as feedstocks, hence, suddenly get a value? This might provide for the ignition sparks for a broader implementation of PHA biopolyesters on industrial scale.

I supremely hope that the investigative and scientific efforts collected and summarized in this *e*Book will inspire researchers all over the world to intensify their activities in this area, and to attract the interest of undergraduates, graduates, as well as of innovative representatives from relevant industrial sectors. Most of all, these accomplishments shall contribute to accelerate the impatiently aspired market break-through of real "bio-plastics" which honestly deserve this designation. This *e*Book underlines that "bio-inspired" solutions for prevailing ecological problems are already developed by experts from the fields of engineering and life sciences, or at least in an advanced status of development; these solutions are waiting for their broad implementation in "White Biotechnology"!

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



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CHAPTER 1

Novel Inexpensive Feedstocks from Agriculture and Industry for Microbial Polyester Production

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Abstract: Polyhydroxyalkanoates (PHAs) are very promising materials which might serve as environmentally-friendly alternative to petrochemical plastics. The main obstacle preventing PHAs from entering the market massively is the final cost of the polymeric material, important portion of which is attributed to carbon substrate. Hence, the researchers have been intensively looking for cheap substrates enabling sustainable production of PHAs. Hence, this chapter summarizes the advances in biotechnological production of PHAs from various waste products originating from industries of different fields, especially from food industry and agriculture.

Keywords: Agricultural waste, Biodiesel industry, Biotechnological production, Carbon-rich waste, Cheese whey, Glycerol, Inexpensive substrates, Lignocelluloses, Lipid substrates, Molasses, Polyhydroxyalkanoates (PHA), Sugar industry, Upstream processing.

GENERAL ASPECTS OF POLYHYDROXYALKANOATE PRODUCTION

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyacids (usually 3-hydroxyacids) which are synthesized by various microorganisms under the conditions of nutrient limitation in the presence of an excessive carbon and energy source. Due to their low solubility and high molecular weight, PHAs do not cause the increase of intracellular osmotic pressure and they are, therefore, ideal storage

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materials. In this role they are much more common storage materials in microorganisms than is glycogen, polyphosphates, or fats, for example [1].

PHAs were discovered by Lemoigne who isolated and characterized poly(3-hydroxybutyrate) (PHB), a member of PHAs family, from *Bacillus megaterium* in 1926. Since this first report the PHAs accumulation has been discovered in many microorganisms including Gram-negative and Gram-positive species (*i.e.*, autotrophs, heterotrophs, phototrophs, aerobes, anaerobes) and archaebacteria. In 1976, Imperial Chemical Industries (ICI Ltd., UK) recognized the potential applicability of PHAs to replace some of the commodity plastics produced from petrochemical resources. Despite the fact that the bacterially produced PHAs were expensive as compared to oil-derived plastics, it was expected that the price of crude oils would rise to high levels, making the production of PHB economically feasible. Because no rise in oil prices occurred, PHB originated as a biodegradable and biocompatible plastic material [2].

Currently, PHAs still attract the attention of many researchers and, therefore, many aspects of their biological function, metabolism, biotechnological production, but also the processing and applications were understood. This chapter aims at providing a detailed insight into the current state of the art PHAs production from inexpensive and/or waste substrates.

Motivation for Utilization of Inexpensive Substrates

The main obstacle preventing the PHAs market from massive expansion is their cost. Generally, PHAs are several times more expensive than their competitors - polymers of petrochemical origin. Principally, currently known technologies of PHA production have three essential disadvantages: i. the use of relatively expensive carbon substrates; ii. the use of aseptic culture of selected or genetically modified strains which require high expenses for the sterilization of equipment and medium, as well as for the maintenance of aseptic conditions during the biosynthesis of bioplastics iii. The use of expensive, often flammable and toxic organic solvents and/or energy- and reagent-consuming methods for the extraction of PHAs from bacterial cells [3].The aim of this chapter is to face the first and the most important problem of industrial PHA production - the cost of entering substrate and the utilization of inexpensive carbon substrates for biotechnological

production of PHAs.

The cost of common substrate (pure sugars or fatty acids) is known to account for almost 50% of the total production costs of PHAs [4]. Therefore, many researchers have been looking for inexpensive substrates enabling economically feasible PHAs production process. Numerous potential substrates, including but not limited to waste products and side products of food industry and agriculture, have been investigated in this perspective. Generally, inexpensive substrates for biotechnological production of PHAs should meet some basic requirement:

a. they should be available in sufficient amount and quality during whole year
b. they should not undergo rapid spoilage by the action of naturally occurring microbes
c. their cost should be sufficiently low even when taking into account the economic aspects of their collection and transportation
d. there should not be other, more profitable technology of their utilization
e. the substrates for PHAs production should not compete with human food chain.

Usually the utilization of inexpensive substrates provides lower PHA yields as compared to processes using pure sugars of fatty acids. Additionally, low overall product yields contribute to the cost, as less efficient bioconversion would require more substrate for the production of the same amount of product. The problem is compounded by the fact that, in most cases, PHA production gives low product concentrations and low overall volumetric productivities. The latter leads to higher capital and operating costs, especially regarding the application of large bioreactor facilities [5].

Hence, despite the fact that the production of PHAs from inexpensive substrates is widely accepted as correct strategy, there are many obstacles which prevent it from large scale industrial applications.

PHA Producing Strains and their Characterization

Polyhydroxyalkanoates are complex class of polyoxoesters which are synthesized by most genera of eubacteria and members of the *Halobacteriaceae* family of the

CHAPTER 2

PHA Biopolyester Production from Surplus Whey: Microbiological and Engineering Aspects

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Abstract: As the lactose-rich aqueous fraction that remains during the cheese production process after separating casein proteins and lipids from whole milk, whey accrues as an industrial by-product at vast quantity at dairies and cheese production manufactories.

The volumes of generated surplus whey almost equal those of the whole milk processed for cheese production, thus by far surmounting the needs of its restricted classical markets such as animal fodder, pharmaceutical matrices, or additives in food production. As a consequence, enormous quantities of whey are contemporarily disposed in aquatic environments, provoking prevailing ecological concern due to its considerable biochemical oxygen demand, and high expenses for the disposal. Hence, sustainable alternatives for a value-added application of surplus whey are needed; contemporarily, biotechnologists in various global regions are questing for such alternatives! Based on the catalytic action of selected microbial production strains, the spare carbon source contained in whey can be converted into value-added bio-products and at the same time, solve the industrial disposal problem.

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Polyhydroxyalkanoates (PHA), a group of biodegradable microbial polyesters with auspicious plastic-like material characteristics, are among the most promising target products accessible from whey´s bio-conversion. Using different production scales, production strains and production modes, excess whey can undergo upgrading to a carbon feedstock for generation of thermoplastic poly(3-hydroxybutyrate) (PHB) homopolyester or, by switching to advanced feeding strategies and application of powerful novel production strains, to tailor-made PHA co- and terpolyesters with customer-oriented material properties. The article covers microbiological, engineering, and economic aspects of whey-based PHA production by meso- and extremophile wild-type or genetically engineered prokaryotic production strains. The route needed to make efforts in this field competitive with current PHA-production on prized feedstocks which in parallel interfere with nutrition for mankind, is presented. This competitiveness is addressed both in environmental and economic aspects.

Keywords: Agricultural waste, β-galactosidase, Carbon-rich waste, Cheese whey, Copolyester, Dairy industry, Dairy waste, Downstream processing, Green energy carriers, Homopolyester, Lactose, Nutraceuticals, Polyhydroxyalkanoates (PHA), Process design, Production strains, Substrates, Surplus materials, Terpolyester, Upstream processing, Whey.

THE NEED FOR VALUE-ADDED AND SUSTAINABLE UPGRADING OF WHEY: HOW TO TURN A SURPLUS PRODUCT INTO A USEFUL RAW MATERIAL

In cheese dairies or casein manufactories, whey accrues as the aqueous fraction that is generated as a side stream after the so called transformation, *i.e.*, the acidic or enzymatic coagulation of milk casein. This liquid fraction represents about 85-95% of the processed milk´s volume [1]; in addition, estimations report that cheese production generates approximately 9 L of whey per kg [2]. Reported amounts of the globally accruing quantities of whey are strongly fluctuating in dependence on the consulted source. Just to visualize the magnitude of generated whey in various countries, we present some selected estimations: While Peters reports an annual quantity of $1.15*10^8$ t [3], Audic estimates about $1.40*10^8$ t per year [4], which is almost twice as high as values reported by Prazers who reports $4.1 * 10^7$ annual tons [5]. Reliable data of OECD-FAO for 2008 are based on the assumption of even $1.60*10^8$ t per year, with an annual rise of 1-2% [1].

Whey accrues as a surplus material in various global regions [6] with highest quantities in North America and Europe. Due to the fact that cow milk is the globally predominant source for cheese production, it is clear that whey of bovine origin represents the lion´s share of the entire whey quantity. In the year 2008, about $4*10^7$ t of whey were generated in the USA, about $5*10^7$ t in the EU, and about $2.2*10^5$ t in Canada, which also constitutes a nation with considerable whey production (values for 1997) [7]. In addition of these countries located in the Northern hemisphere, also southern countries produce increasing volumes of whey; as a representative estimation, 270,000 tons annually are reported to accrue in India as a pure surplus stream without any use for further application [8].

On the one hand, the market for products containing whey include nutritionally relevant products *e.g.*, candies, whey powder used as dietary supplement for increasing the muscle size of body builders, drinks based on whey, food processing additives (*e.g.*, for manufacturing of ice cream or meat processing), chocolate substitute, infant food, cosmetics (*e.g.*, whey-containing shower gels, body lotions, or face crèmes) as well as products used for pharmaceutical purposes. However, due to its nature, these applications are restricted [8]. On the one hand, it has to be considered that whey *per se* has quite a lot of nutritive value based on its richness in proteins, carbohydrates, lipids, vitamins, and diverse essential minerals such as calcium (Table **1**); 100 g of consumed whey corresponds to an energy uptake exceeding 100 kJ. Further, a high number of people, about 75% of adults worldwide, suffer from hypolactasia, also known as lactose intolerance, due to insufficient β-galactosidase (lactase) activity [9]. This lacking lactase activity, also referred to as hypolactasia, can result in hypolactasia still during later life, which mainly occurs in countries of the Southern hemisphere and South European regions [10]. This frequent occurrence of deficient lactase activity impedes a broader application of whey for human nutrition.

Recent reports underline the need for new, innovative markets to valorise whey; at the end of July 2015, whey powder prices in the EU dropped to their lowest level since November 2010, only from March 2015 to July 2015, the prizes decreased by 28%, *inter alia* caused by a reduced export of whey powder to China [11]. This goes in parallel with the contemporary debate about the excessive amounts of milk which are on the European market; a valorisation of whey as the major by-

CHAPTER 3

Biopolyesters and Related Valuable Products in Phototrophic Microbes

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Abstract: Poly(3-hydroxyalkanoates) are widely used environmentally sound bioplastic produced by numerous microbes including anoxygenic and oxygenic photosynthetic bacteria. Similarly to heterotroph microbes, the photosynthetic microbes can accumulate these polyester-type storage materials under nutrient limitation such as nitrogen, phosphorous or sulfur starvation. A number of purple non-sulfur, sulfur photosynthetic and cyanobacteria can produce PHA of various compositions. The amount, type, length and monomer composition of the PHA strongly depend on the nutrients and the growth conditions, and the light intensity also seriously affects the yield. While anoxygenic photosynthetic microbes need external inorganic or organic electron source, for photosynthetic CO_2 fixation, the oxygenic microbes, cyanobacteria can gain electron from water splitting. In principle, anoxygenic photochemo-litoautotrophic and cyanobacteria can produce PHA from CO_2 as sole carbon source, but addition of organic substrates significantly improves the yield. PHA can also be decomposed by the cells under certain conditions and the PHA metabolism has connections to other metabolic routes including glycogen metabolism. There are several approaches for improving the production rate and yield and – for this purpose –

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a number of artificially genetically modified microorganisms, metabolic routes were constructed.

Keywords: Bacteria, Biocompatible bioplastics, CO_2 fixation, Cyanobacteria, Microbial storage materials, Photosynthetic microbes, Polyhydroxyalkanoates (PHA), Purple photosynthetic bacteria.

INTRODUCTION

In unbalanced growth conditions with very frequent substrate fluctuations a widespread strategy is to store surplus substrates. In microorganisms, the accumulation of certain compounds offers selective benefits when the growth conditions are not optimal. For example, zero-valence sulfur, glycogen, polyhydroxyalkanoates (PHA) and polyphosphate can be accumulated depending on the growth conditions [1]. PHA represents carbon and energy storages in more than 300 Gram-positive, Gram-negative bacterial and archaeal species [2].

These polymers are synthesized by the microbes during impaired cultivation conditions, when the cell proliferation is hampered by a nutrient limitation (generally nitrogen, phosphate, or oxygen).

More than 150 hydroxyalkanoic acid monomers are involved in PHAs compositions (Fig. **1**) [3]. PHAs can be grouped into two classes according to the number of carbon atoms in the monomers: short chain length PHAs (*scl*-PHA), containing 3 to 5 carbon atoms in the monomer and medium chain length PHAs (*mcl*-PHA), synthesized from monomers composed of 6 to 15 carbon atoms.

PHA synthesis has been an important field of biotechnology for over three decades by now [4]. Since the feasibility of the technology depends on the economic benefits, all factors influencing the profitability are to be considered. The cost of the raw materials may total up to more than half of the production costs of PHA [5], therefore bacteria growing on cheap substrates present an evident route in searching for viable technologies.

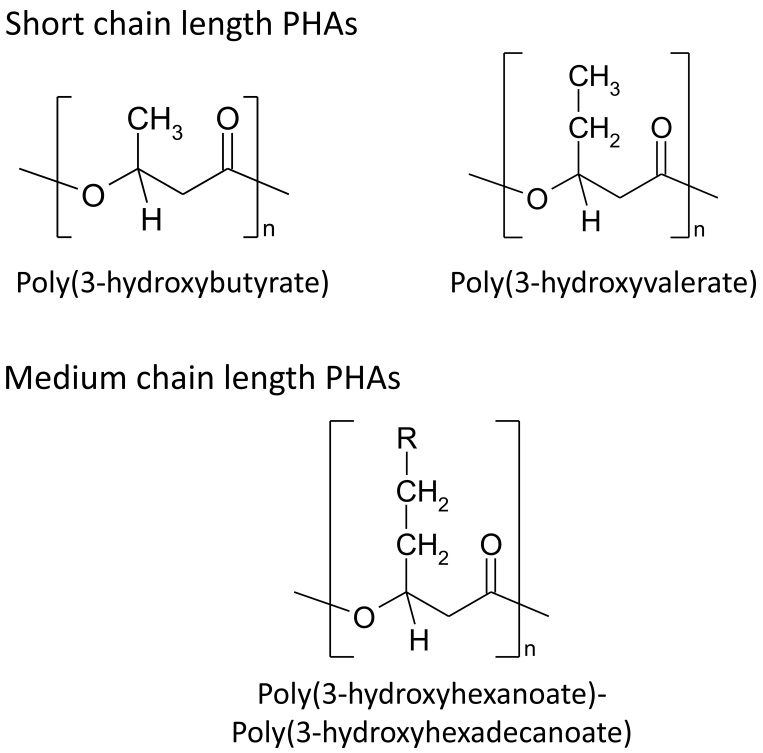

Fig. (1). Chemical structure of the short and medium chain PHAs. The R alkyl group is built up from 1-11 carbons.

Although, most of the studies focus on the PHA production by non-photosynthetic bacteria (for recent reviews see [6 - 9]), both anoxygenic and oxygenic photosynthetic bacteria can also produce and decompose PHA. While in heterotrophic fermentative bacteria, the cells produce PHA from various cheap substrates, such as: sugars, starch, (hemi)cellulose, whey, oil and glycerol, photochemolitoautotrophic microbes and cyanobacteria can utilize CO_2 for PHA production, although the yield can be substantially improved by supplementing the media with organic compounds including volatile organic acids. Nevertheless, CO_2 would be the cheapest and environmentally best substrate for production of polyester bioplastics. Therefore, in the following section, the current status of the research on production of PHA by photosynthetic microbes is overviewed.

CHAPTER 4

Biopolymer Production by Mixed Microbial Cultures: Integrating Remediation with Valorization

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Abstract: Polyhydroxyalkanoates (PHA) production by mixed microbial cultures (MMC) became the central point of many research works. Firstly discovered in wastewater treatment systems, namely those related with biological phosphorus removal, it became a process to be considered for polymer production intended to replace petrochemical derived plastics. The ability to store PHA provides microbial communities a competitive advantage for survival under transient conditions typical of waste treatment systems. In this way, MMC can continuously adapt to the operational conditions increasing the number PHA-storing organisms with minimum requirements of sterility. MMC can produce PHA using waste or industrial by-products as substrates, allowing for their valorization. The use of waste, together with the lower requirements in sterility and process control, could signify a decrease on PHA production costs. The price is one of the main drawbacks that prevent the increase of world PHA market share. This chapter intends to contextualize the use of MMC for PHA production, by describing the different approaches and compare them with the traditional processes employing pure or genetically modified organisms. The main processes to select PHA-storing MMC are discussed, namely alternating of anaerobic and aerobic (AN/AE) or

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anoxic (AN/AO) conditions and aerobic dynamic feeding (ADF). The use of wastes as substrates, the operational conditions, the microorganisms involved as well as the molecular methods used for their identification are also the focus of this work.

Keywords: ADF, AN/AE, Mixed microbial cultures, Molecular methods, Operational conditions, Polyhydroxyalkanoates, Selection, Valorization, Waste, Waste treatment.

INTRODUCTION

Mixed Culture Definition

It is common knowledge that polyhydroxyalkanoates (PHA)-producing genera are quite ubiquitous in nature. So far, more than 300 were found and part of them cultivated but it is believed that a larger number still remains unknown [1, 2]. Until now, the search for pure and defined cultures with a high PHA-storing capacity has been the objective of most of the researchers working in this field. However, in nature, microorganisms live in community with different relationships among them. Most of the times, the isolation of bacteria with the desirable characteristics is quite difficult. It is believed that more than 99% of microorganisms have not been successfully cultivated yet and, surely, among this number many PHA-producing strains can be found [3]. For the majority of bacteria, the ability of accumulating internal reserves represents a major competitive advantage to survive in natural environments. Carbon-based internal reserves as PHA and glycogen or external like some polysaccharides, as well phosphorus (namely, polyphosphate - PolyP), nitrogen (cyanophycins) or sulfur-based reserves, are usually advantageous for the producing organisms and were found in many different species of bacteria, including those that constitute activated sludge populations, the most known and studied mixed microbial culture (MMC). Activated sludge is a complex consortium of several populations of microorganisms of undefined composition, which depends on feeding and process conditions. Other MMCs can be found in open biological systems that appear in natural environments, as soil, seawater, sediments, rumen, N_2-fixing bacteria, or, as activated sludge, in human created environments under non-sterile conditions, namely ponds, waste storage facilities, *etc*.

Historical Background

Due to its role in wastewater treatment plants (WWTPs), the first MMC to be studied was activated sludge. In early 70s, the presence of PHA granules inside cells belonging to an activated sludge community was observed for the first time [4]. Later on, PHA were found to play a key-role on the competition between polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) for the external carbon source in enhanced biological phosphorus removal (EBPR). In this type of systems, PHA are stored when the external carbon source, usually a short-chain organic acid (SCOA), is supplied in the absence of an external electron sink. The lack of electron sink prevents microbial growth but PHA-storing bacteria are able to store the external carbon source as intracellular reserves to be used as carbon and energy source in the absence of external substrate and/or an electron sink is available [5]. In this way, PAOs and GAOs were the first organisms present in MMC to be described as PHA-storing bacteria. Later on, a correlation between PHA storage and use and the phenotypic identity of PAOs and GAOs was established by using fluorescence *in situ* hybridization (FISH) and postFISH nile blue staining [6]. The first attempts to identify the microorganisms involved in EBPR, including both PAOs and GAOs, started almost 40 years ago, but so far no attempts were successful. Potential methods to successfully isolate these bacteria were tested through the use of *e.g.* micro-manipulation techniques from enriched cultures of these organisms [7]. In the following years, PHA production by MMC systems based on the alternation of availability of electron sink was developed, being the most successful those where GAOs were dominant [8].

Bulking was another aspect of activated sludge correlated with PHA storage observed in aerobic WWTP [9]. The process configuration with alternation of excess of carbon source periods with depletion periods was observed to disfavor the selection of filamentous bacteria responsible for the bulking phenomena and select floc-formers with enhanced PHA storage capacity [9]. The observations of Majone *et al.* (1996) confirmed the hypotheses raised by Daigger and Grady (1982) to explain the phenomenon. After a period of absence of external carbon source, during which the levels of enzymes and RNA required for growth decreased, a sudden increase of external substrate triggered the storage of internal

CHAPTER 5

Ralstonia Eutropha and the Production of Value Added Products: Metabolic Background of the Wild-Type Strain and its Role as a Diverse, Genetically-Engineered Biocatalyst Organism

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Abstract: *Ralstonia eutropha* (a.k.a., *Cupriavidus necator*) is at the forefront of the research movement towards sustainable bioproductions. There are two principal reasons for the popularity of *R. eutropha* in this arena: 1) the organism has a versatile metabolism and can utilize a wide variety of carbon sources from sugars and lipids to carbon dioxide and aromatic compounds for growth and fermentative bioproductions, 2) it can store a large amount of carbon as intracellular polymer. *R. eutropha* is known as the model system for polymer (polyhydroxyalkanoate) biosynthesis and mobilization. Many valuable works have been published on this specific topic. In the recent years, however, *R. eutropha* is being used as a host organism for heterologous bioconversions, such as biofuel synthesis. Again, researchers are exploiting the metabolic versatility of the organism to create novel products and processes. This chapter chronicles the key discoveries of *R. eutropha* biology and presents the biomanufacturing context that inspired many research groups to tailor the organism as a novel biocatalyst.

Keywords: Autotrophy, Biofuel, Biopolymer, Bioremediation, *Cupriavidus necator*, Fed-batch cultivation, Fermentation, Isobutanol, Medical products, *M*etabolism, Polyhydroxyalkanoate (PHA), *Ralstonia eutropha*.

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INTRODUCTION

In the early 1800s, the French gastronome Jean-Anthelme Brillat-Savarin said "Tell me what you eat, and I'll tell you what you are." This adage, when applied to the world of microorganisms, is most poignant, as many bacteria are defined and even named for the substrates from which they derive nutrition. Indeed, it is possible that Brillat-Savarin was familiar with the 1676 discoveries by Antonie van Leeuwenhoek of "animalcules" [*i.e.,* bacteria] in many different environmental niches. Nowadays, we understand that these microbes populate their respective niches due to the availability of usable nutrients. Since the advent of microbial ecology and subsequent physiological characterization, microbiologists seek to classify bacteria in part based on the compounds they are capable of eating.

Ralstonia eutropha, also named *Cupriavidus necator*, is a Gram negative bacterium that is found in soil and aquatic environments. The species name "*eutropha*" can be defined loosely as "good eating." Indeed, nutrition and carbon conversion have defined the bacterium almost since its discovery in 1952 [1]. *R. eutropha* is a master carbon-utilizer and carbon-converter, and this attribute has defined its role as a model organism for producing biotechnologically relevant products like polyhydroxyalkanoate (PHA) polymers. This bacterium is capable of growth in many different niches: in the presence or absence of O_2, in a chemoautotrophic environment, and, obviously, in a heterotrophic environment. It is this versatility that has made *R. eutropha* a microbial chassis for bioconversion of cheap carbon (waste) to value added compounds.

A Brief History of *R. eutropha* (*C. necator*): What's in a Name?

In 1952, Albert Schatz and Carlton Bovell isolated a bacterium from "lawn soil" that exhibited hydrogenase activity. The isolated bacterium was also shown to assimilate a variety of organic acids [*e.g.,* acetate, α-ketoglutarate]. Most notable about this find was the highly active hydrogenases expressed by the organism and, to Schatz and Bovell, the ease of which the organism could be manipulated. To the latter end, the newly isolated organism was named *Hydrogenomonas facilis*, "because of the ease with which it lent itself to experimentation" [1, 2]. The same

organism was being characterized in Europe by Wilde and coworkers and was named *Hydrogenomonas eutropha* because of its ability to grow robustly on a variety of carbon sources, including CO_2 [3]. In fact, the prototypical wild-type strain, H16, was isolated by Wilde. Almost a decade later, *H. eutropha* was renamed *Alcaligenes eutrophus* because of its physiological indicators as a rod-shaped denitrifying bacterium that produces no pigment [4]. It had been suggested that the name *Hydrogenomonas* was illegitimately applied and *A. eutrophus* was listed in the Approved List of Bacterial Names [4, 5]. In 1995, the name was changed again to *Ralstonia eutropha*. It was determined that select species from the genus *Burkholderia* and *Alcaligenes* were phenotypically similar to each other and, with 16S rRNA/rDNA sequence comparison data, were determined to fit best in the genus *Ralstonia* [5]. This classification stood until 2004, when a new genus *Wautersia* was established and *R. eutropha* was then classified as *Wautersia eutropha*. The *Wautersia* lineage was established mainly as a result of re-analysis of 16S rRNA/rDNA sequences, and *R. eutropha*'s renaming was a result of a previous observation of two distinct sub-classes of *Ralstonia* [6]. The *W. eutropha* classification lasted less than a year when it was renamed *Cupriavidus necator* as a result of DNA-DNA hybridization experiments. The authors of the study, Vandamme and Coenye, cited Rules 15, 17, 23a and 37a [1] of the International Code of Nomenclature of Bacteria [7]. It should be noted that members of the genus *Cupriavidus* tend to exhibit resistance to metals (Hence the first part of the name *Cupriavidus*, as in copper]. Indeed, several strains of *R. eutropha* (*C. necator*) have demonstrated resistance to metals like nickel, cadmium and others [8]. There have also been found putative metal tolerance genes in the genome sequence of *R. eutropha* strain H16 [9]. Vandamme and Coenye state that there can be only one correct name for a bacterial species, according to the International Code of Nomenclature of Bacteria [7, 10]. They go on to state that the correct genus and species name for *R. eutropha* should be *Cupriavidus necator*, since the latter was established in 1987, well before either the *R. eutropha* or *W. eutropha* names were designated [7]. Thus, *C. necator* has become a generally accepted name for the organism, and, to date, no further name changes appear to have been suggested. However, it should be noted that several pioneering researchers, namely the Sinskey and Steinbüchel groups and offshoots of those groups, have elected to continue use of the *R. eutropha* name and still publish findings based on

CHAPTER 6

PHBHV Biosynthesis by *Haloferax mediterranei*: from Genetics, Metabolism, and Engineering to Economical Production

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Abstract: The halophilic archaeon *Haloferax mediterranei*, has shown promise for the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV), a desirable bioplastic, from many low-cost carbon sources. To obtain a full understanding of PHBHV biosynthesis and to provide novel strategies for polyhydroxyalkanaote (PHA) production by haloarchaea, the complete genome of *H. mediterranei* has been determined, and a genome-wide investigation of the genetics and metabolism of this haloarchaeon involved in PHBHV biosynthesis was performed in the past few years. This exploration covers the identification of key genes for PHBHV biosynthesis, the specific precursor supplying pathways, the PHA granule modulation, and PHBHV mobilization, as well as its linkage to haloarchaeal-specific acetyl-CoA and propionyl-CoA assimilation. These advances have not only revealed many haloarchaeal-specific enzymes and pathways for PHBHV biosynthesis, such as the archaeal-type PHA synthases, the haloarchaeal-type β-ketothiolases with two distinct subunits, and the novel 3-hydroxypropionate pathway coupling CO_2 fixation into PHBHV, but have also provided strategies for bioengineering haloarchaea for economical production of PHBHV or tailor-made higher-value PHA. This chapter will summarize these current progresses of PHBHV biosynthesis by *H. mediterranei*, thus providing a perspective for economical production of industry-scale or higher-value PHAs by haloarchaea in the near future.

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Keywords: Acetoacetyl-CoA reductase, Acetyl-CoA, β-ketothiolase, β-oxidation, Carbon sources, Copolyester, Economical production, Genes, Genome, *Haloferax mediterranei*, Halophilic archaea, PHA biosynthesis, PHA depolymerase, PHA granule, PHA mobilization, PHA synthase, Polyhydroxyalkanoates (PHA), Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV), Propionyl-CoA, Regulation, Tailor-made PHA, Terpolyester.

INTRODUCTION

Haloferax mediterranei, previously known as *Halobacterium mediterranei*, was first isolated from seawater evaporation ponds near Alicante, Spain [1, 2]. *H. mediterranei* is a pleomorphic rod-shaped, slightly motile halophile belonging to the newly proposed *Haloferacaceae* family (moved from the *Halobacteriaceae* family) of the domain Archaea [3, 4]. The extracellular polysaccharides produced by *H. mediterranei* display xanthan-like properties and give the carotenoid-rich red colonies a typical mucous character on agar plates [5]; the strong coloration, based on C50-carotenoids pigments, is also easily visible in liquid cultures of this organism (Fig. **1**).

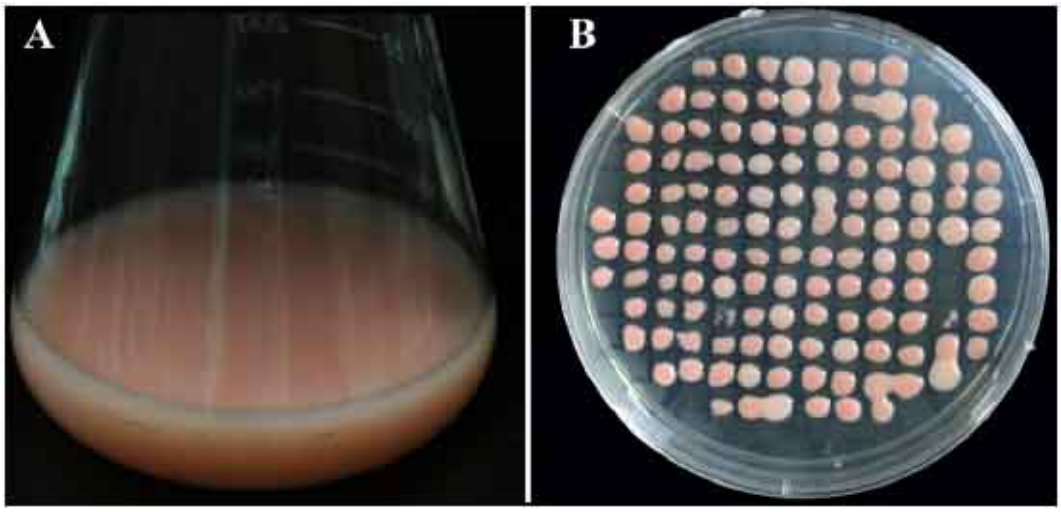

Fig. (1). Culturing of *H. mediterranei* in liquid media (A) and on agar plate (B).

H. mediterranei is strongly amylolytic and lipolytic, and it can use a variety of carbohydrates, polyalcohols, organic acids, or amino acids as a sole source of

carbon and energy. It grows faster than most other members of *Halobacteria* (1, 2). It can grow aerobically over a wide range of salt concentrations (optimum at 20% and survival above 3% total salts, including 20-40 mM Mg^{2+}) with an optimal temperature of 37-51 ºC and pH-value of 6.5 to 7.2. *H. mediterranei* can also grow anaerobically by reducing nitrate and nitrites to N_2 [2, 6]. *H. mediterranei* was observed to fix CO_2 in the form of HCO_3^-, which might also be an important carbon source for *H. mediterranei* at high salt concentration [7 - 10].

In stationary phase or upon other stress conditions, *H. mediterranei* can produce halocins [11], gas vesicles [12, 13], and accumulate polyhydroxyalkanoate (PHA) [14]. This makes it an interesting model haloarchaeon for the research of haloarchaeal physiology. The PHA produced by *H. mediterranei* was determined as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) [15], a desirable bioplastic with multiple applications. The genome of *H. mediterranei* has already been determined, and the genetic manipulation tools have also been well-established [16, 17]; thus, *H. mediterranei* is a potential novel microbial cell factory for PHBHV production.

In this chapter, we will summarize recent progress from genetics, metabolism, and bioengineering of *H. mediterranei* for PHBHV biosynthesis to tailor their properties, and to make the production of PHBHV by this haloarchaeal cell factory more economic.

THE GENOME OF *H. MEDITERRANEI*

The complete genome sequence (3,904,707 bp) of *H. mediterranei* was determined in 2012 by a combination of 454 pyrosequencing and Sanger sequencing, which revealed four circular replicons of the genome [17], including one chromosome and three mega-plasmids named pHM100, pHM300 and pHM500 (Table **1**). The genome encodes 3,864 putative proteins, 57 tRNAs, and two 5S-16S-23S rRNA operons. Many important genes involved in PHBHV biosynthesis are located in the mega-plasmid pHM300 (Fig. **2**).

CHAPTER 7

Bacterial Genetic Modifications for Improving Polyhydroxyalkanoates Production from Inexpensive Carbon Sources

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Abstract: There are several phases of the PHA production chain where genetic tools may help, starting from the microbial strain isolation and characterization, through all the production steps, until the downstream processes.

Leaving aside the huge number of genetic modifications accomplished on many bacterial species to clarify basic biochemical, genetic and metabolic aspects of PHA metabolism, several interesting attempts have been reported aimed at improving the performance of the microorganisms showing potentiality for a possible production process.

Most of the available information deals with the use of genetic tools for (i) characterization and identification of new isolates, (ii) testing new isolates for the presence of relevant genes involved in PHA production/degradation, (iii) metagenomic approach in complex environments for searching relevant genes (and strains), (iv) assessment of PHA genes expression and control of depolymerase genes, (v) metabolic engineering of producing and not producing bacterial strains, (vi) improving downstream processes, (vii) making PHA producing strains able to utilize different carbon sources (simple sugars, fats, starch-lignocellulose, other complex substrates), (viii) cloning PHA genes in other recipient bacteria.

Due to the huge amount of results published in each of the above fields, after a general overview on the methods used to search for new PHA producing microbial strains, this

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chapter will try to summarize the most relevant results obtained through genetic engineering tools for the production of PHAs from cheap carbon sources in view of possible industrial applications.

Keywords: Bacteria, Cloning PHA genes, Enzymes, *Escherichia coli*, Genes, Genetic engineering, Genetic modification, Inexpensive carbon sources, Inexpensive substrates, Organic by-products, PHA production, Polyhydroxyalkanoates (PHA), Unrelated carbon sources.

TRADITIONAL AND DNA-BASED METHODS TO LOOK FOR PHA PRODUCERS

The accumulation of polyhydroxyalkanoates (PHAs) as energy and carbon storage is a widespread trait among several dozens of Eubacteria and Archaea [1]. PHA can be obtained starting from various in-excess carbon sources; *Cupriavidus necator* (formerly known as *Wautersia eutropha, Ralstonia eutropha* and *Alcaligenes eutrophus,* see below) is the most widely studied microorganism for the production of PHA but other strains, belonging to species such as *Bacillus cereus* and *B. megaterium, Sinorhizobium meliloti, Azotobacter chroococcum*, *Pseudomonas putida* or *Haloferax mediterranei*, were also evaluated for PHA production from various and low-cost carbon sources such as sugars (glucose, maltose, lactose, xylose *etc.*), alkanes (*e.g.* exane, octane *etc.*), alkanoic acids (*e.g.*, acetic, propionic, butyric, valeric, oleic acids *etc.*), alcohols (*e.g.*, ethanol, methanol, glycerol *etc.*) or gases (carbon dioxide and methane) [2 - 5].

Most of the PHA producing strains have been deposited in culture collections such as ATCC or DSMZ, making them commercially available. An exhaustive review of PHA producing microorganisms in culture collections have been recently proposed [6]. Usually genetic and biochemistry of deposited strains in terms of carbon utilization and PHA accumulation, are well known and documented in scientific papers. These knowledges greatly facilitated a quick start-up of research and the following industrial production.

Unfortunately, although the ability to synthesize PHAs is a widespread trait among microorganisms, it is true that not all of these bacteria are suitable for an industrial production due to their lacking of robustness and inability to survive to

the highly stressing fermentation conditions [7]. Although to solve these problems a series of optimization strategies have been attempted, such as developing better bacterial strains or supplying renewable low-cost carbon substrates, it is undoubting that the isolation and identification of suitable PHAs producing microorganisms from the environment are key factors for PHA-production processes. Therefore, intensive research was conducted aimed to discover, characterize and improve newly isolated bacterial strains, able to produce huge amount of PHAs from different and unrelated carbon sources, to survive in environments extreme for temperature, pH-value or salinity, and to convert residual and low-cost carbon sources.

Testing New Isolates for the Ability to Produce PHA

Traditional Methods

The most common traditional technique available for analysis of PHAs in bacterial cells is gas chromatography (GC) that has been proposed by Braunegg and colleagues 40 years ago [8] and later refined [9]. The GC method involves hydrolysis and subsequent methanolysis or propanolysis of the PHAs in whole cells in the presence of sulfuric acid and chloroform. Although this method is time-consuming, involves extensive use of solvents and does not enable to test large number of strains in short time, GC analyses still remain the final confirmation technique but cannot be considered a screening methods.

The utilization of phase-contrast light microscope allows to appreciate highly refractive PHA granules directly inside bacteria in a fresh culture, even if it is often difficult to distinguish PHA from other intracellular inclusions such as spores or glycogen. The application of lipophilic dyes such as Sudan black B, Nile blue A, and Nile red is today the “first line screening” of PHA containing bacteria for its easiness: these colorants show in fact a high affinity for lipophilic materials and therefore can stain hydrophobic PHA [10 - 14].

The oxazine dye Nile blue A and its fluorescent oxazone form, Nile red, can be directly included in the medium and used as a simple, specific and highly sensitive staining method to detect poly(3-hydroxybutyric acid) and other polyhydroxyalkanoic acids in growing bacterial colonies, into the culture broth or

CHAPTER 8

Medium-Chain-Length Poly-3-hydroxyalkanoates

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Abstract: Medium-chain-length poly-3-hydroxyalkanoates (*mcl*-PHAs) were discovered in the 1980s, 60 years after their more famous biopolyester ancestor poly--hydroxybutyrate. Accumulated only by a group of related *Pseudomonas* species, these polyesters are remarkable in their structural diversity and applications. *mcl*-PHAs are highly water resistant, yet readily susceptible to enzymatic degradation. They can be used in packaging, paints, toners, adhesives and in many biomedical applications but have yet to be produced commercially. *mcl*-PHAs are most efficiently produced from carboxylic (fatty) acids such as octanoic acid but can also be produced from many non-related substrates such as glucose. Recent advances in fermentation and separation processes, as well as strain development are improving polymer quality and reducing production cost, which should soon lead to the adoption of *mcl*-PHAs by industry.

Keywords: Applications, Biosynthesis, *mcl*-PHA, Medium-chain-length PHA (*mcl*-PHA), Poly-3-hydroxyalkanoate, Production, Recovery, Thermoelastomer.

INTRODUCTION

This chapter is focused on different types of polyesters including some that are biologically produced. Although they all contain subunits linked by an ester bond, polyester properties vary greatly. For example, both poly-3-hydroxyalkanaotes (PHAs) and polylactides (PLAs) are polyesters that degrade in the natural environment, but PLAs are polymerized in an *ex vivo* chemical process and

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degrade *via* chemical hydrolysis while PHA degradation occurs *via* extracellular enzymes. Among the PHAs (Fig. **1**), the short-chain-length (scl)-PHAs have less than three carbon atoms in the R group while medium-chain-length (*mcl*)-PHAs have three or more carbon atoms in the R group. *mcl*-PHA films may absorb water, but the rate of hydrolysis near neutral pH-values is very slow [1]. *mcl*-PHAs are unique in many ways. In contrast to most *scl*-PHAs, they are thermoelastomers and are produced by only certain species of the genus *Pseudomonas*. *mcl*- and *scl*-PHAs are degraded by different types of depolymerase enzymes. Both *mcl*- and *scl*-PHAs can be produced from glucose but the biosynthetic pathways are distinct and the thermomechanical properties of the resulting materials (*e.g.*, poly-3-hydroxydecanoate (PHD) and poly--hydroxybutyrate (PHB)) are strikingly different. This chapter will examine the production and possible applications of *mcl*-PHAs focusing on their relationship to other bioplastics, especially *scl*-PHAs.

DISCOVERY AND POTENTIAL APPLICATIONS

While studying *Bacillus megaterium* in the 1920s, Maurice Lemoigne identified and began the characterization of the first and most ubiquitous PHA, PHB [2]. He proved that it was a high molecular weight polyester long before the pioneering work of Carothers [3] on polyesters produced *via* chemical synthesis and only shortly after Hermann Staudinger proposed the existence of covalently bonded macromolecules.

Baptist examined the thermomechanical properties of PHB and developed applications as a thermoplastic during the 1960s, especially as sutures [4], but found that it suffered from limitations as a thermoplastic, particularly thermal instability. As a homopolymer, its physical properties are limited. For use as a thermoplastic, PHB needs to be melted, but degrades at its melting temperature T_m and loss of molecular weight further deteriorates its mechanical properties. The breakthough in application of PHAs as thermoplastics came from the realization that the *scl*-PHA polymerases could polymerize other monomers than 3-hydroxybutyrate. Most naturally occurring *scl*-PHAs are PHB homopolymers since many, if not most, environmentally available carbon sources such as glucose

are metabolized to acetate, the building block for 3-hydroxybutyrate synthesis. However, it was found that feeding propionic or valeric acid to *Alcaligenes eutrophus* (now known as *Cupriavidus necator* and previously named *Wausteria eutropha* and also *Hydrogenomonas eutrophus*) produced 3-hydroxyvalerate which is also a good substrate for *scl*-PHA polymerases. Incorporation of subunits other than HB reduces crystallinity which lowers the melting point and increases flexibility. However, even with this discovery, the thermomechanical properties of these *scl*-PHAs are limited.

Although they were also reported as bacterial inclusions, until the early 1980s *mcl*-PHAs existed unidentified by the bacteriologists studying these organisms. Indeed, Stanier in his massive treatise on Pseudomonad taxonomy [5], and adopted by Bergy's Manual of Systematic Bacteriology to classify pseudomonads, described these *Pseudomonas* species as "do not accumulate poly-β - hydroxybutyrate as a cellular reserve material, and are unable to attack this polymer by means of extracellular enzymes". *mcl*-PHA emerged as distinct group of PHA polyesters only three decades ago. Perhaps in a similar manner to Lemoigne, researchers in the Witholt lab were working on medium-chain-length alkane (*e.g.*, octane and 1-octene) utilization by *P. oleovorans* and wondered at the composition of the inclusion bodies [6]. They isolated the granules, expecting PHB, but instead found another 3-hydroxy polyester composed primarily of 3-hydroxyoctanoate. It was eventually named *mcl*-PHA by the discoverers. This was a wise decision as its synthesis requires enzymes able to utilize medium-chai--length carboxylic acids and since even longer chain PHAs have been discovered [7]. It was soon understood that most, if not all ribosomal RNA type 2 *Pseudomonas* species can accumulate *mcl*-PHA, but not *scl*-PHAs. PHA composition from specific carbon substrates depends mostly on the nature of the synthase. The synthase responsible for *mcl*-PHA production in pseudomonads is now classified as type 2.

There are now well over 100 different known subunits, all with structures fitting the formula of (Fig. **1**). They can be incorporated into *mcl*-PHAs due to the broad substrate specificity of the polymerases used in their synthesis. Some of the side-chains of these subunits are chemically reactive, so further diversity may be obtained through chemical modification, including grafting to other biomaterials

SUBJECT INDEX

A

B

“This book gives an excellent overview on biotechnological aspects of polyhydroxyalkanoate production ranging from sustainable feedstocks, novel methods to identify PHA producers, traditional and genetic engineering of strains, phototrophic and mixed culture cultivations, downstream processing to finally a vast range of material properties. I enjoyed reading and highly recommend this book to both newcomers and experts who want to learn more about this fast growing and exciting field of biodegradable plastics.”

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

Martin Koller

Martin Koller was awarded his PhD degree from Graz University of Technology for his thesis on polyhydroxyalkanoate (PHA) production from dairy surplus streams which was enabled by the EU-project WHEYPOL (“Dairy industry waste as source for sustainable polymeric material production”), coordinated by Gerhart Braunegg, one of the most eminent PHA pioneers. As senior researcher, he worked on bio-mediated PHA production, encompassing development of continuous and discontinuous fermentation processes, and novel downstream processing techniques for sustainable PHA recovery with a research focus on cost-efficient PHA production from surplus materials by bacteria and archaea. He holds numerous articles in high ranked scientific journals, authored several chapters in scientific books, gave plenty of invited and plenary lectures at conferences, and supports the editorial teams of distinguished journals. Later, he coordinated the EU-FP7 project ANIMPOL (“Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products”), investigating the conversion of animal processing industry´s waste streams towards structurally diversified PHA and follow-up products. In addition to PHA exploration, he was also active in microalgal research and in biotechnological production of various marketable compounds from renewables by yeasts, chlorophyte, bacteria, archaea, fungi or lactobacilli.