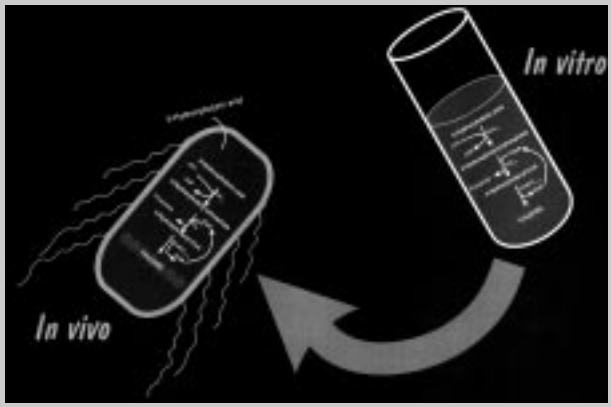
Review: This article provides an overview of biopolymers, classed according to their chemical structures, function and occurrence, the principles of biosynthesis and metabolism in organisms. It will then focus on polyhydroxyalkanoates (PHA) for which technical applications in several areas are currently considered. PHAs represent a complex class of bacterial polyesters consisting of various hydroxyalkanoic acids that are synthesized by bacteria as storage compounds for energy and carbon if a carbon source is present in excess. Poly(3-hydroxybutyrate), poly(3HB), is just one example. Most other PHAs are only synthesized if pathways exist which mediate between central intermediates of the metabolism or special precursor substrates on one side and coenzyme A thioesters of hydroxyalkanoic acids, which are the substrates of the PHA synthase catalyzing the polymerization, on the other side. During the last decade, basic and applied research have revealed much knowledge about the biochemical and molecular basis of the enzymatic processes for the synthesis of PHAs in microorganisms. The combination of detailed physiological studies, utilization of the overwhelming information provided by the numerous genome sequencing projects, application of recombinant DNA technology, engineering of metabolic pathways or enzymes and molecular breeding techniques applied to plants have provided new perspectives to produce these technically interesting biopolymers by novel or significantly improved biotechnological processes or by agriculture. Some examples for successful *in vivo* and *in vitro* engineering of pathways suitable for the synthesis and biotechnological production of PHAs consisting of medium-chain-length 3-hydroxyalkanoic acids and short-chain-length hydroxyalkanoic acids will be provided.



Integration of an *in vitro* engineered poly(3HB) biosynthesis pathway into the metabolism of *E. coli*.

Perspectives for Biotechnological Production and Utilization of Biopolymers: Metabolic Engineering of Polyhydroxyalkanoate Biosynthesis Pathways as a Successful Example

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I Occurrence, Structures, Biosynthesis Principles and Functions of Biopolymers

Living matter is able to synthesize a wide range of different polymers, and in most organisms these biopolymers contribute the major fraction of cellular dry matter. The functions of biopolymers are, in most cases, essential for the cells and are as manifold as their structures.[1]

Chemical Classes of Biopolymers

Organisms are able to synthesize an overwhelming variety of polymers which can be distinguished into eight major classes according to their chemical structure: (i) nucleic acids, (ii) polyamides such as proteins and poly(amino acids), (iii) polysaccharides, (iv) organic polyoxoesters such as poly(hydroxyalkanoic acids), poly(malic acid) and cutin, (v) polythioesters, which were only reported recently,[2] (vi) inorganic polyesters with polyphosphate as the only example, (vii) polyisoprenoides such as natural rubber or Gutta Percha and (viii) polyphenols such as lignin or humic acids (Table 1).

Functions of Biopolymers

These biopolymers fulfil a range of quite different essential functions for the organisms such as conservation and

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Table 1. Seven classes of biopolymers: characteristics of their biosynthesis and occurrence.

Class		Template-dependent synthesis	Substrate of the polymerase	Synthesis in Prokaryotes	Synthesis in Eukaryotes
1. Polynucleotides	Nucleic acids	yes	dNTPs, NTPs	yes	yes
2. Polyamides	Proteins	yes	Aminoacyl-tRNAs	yes	yes
	Poly(amino acids)	no	Amino acids	yes	yes
3. Polysaccharides		no	Sugar-NDP, Sucrose	yes	yes
4. Polyoxoesters		no	Hydroxyacyl coenzyme A	yes	(no)
5. Polythioesters		no	Mercaptoacyl coenzyme A	yes	no
6. Polyphosphate		no	ATP	yes	yes
7. Polyisoprenoids		no	Isopentenylpyrophosphate	no	plants, some fungi
8. Polyphenols	e. g. Lignin	no	Radicalic intermediates	no	only plants

expression of genetic information, catalysis of reactions, storage of carbon, energy or other nurients, defending and protecting against the attack of other cells, hazadous environmental factors, sensing of biotic and abiotic factors, communication with the environment and other organisms, mediation of the adhesion to surfaces of other organisms or of non-living matter and many more. Alternatively, they may be structural components of cells, tissues and entire organisms. To fulfil these different functions, biopolymers must exhibit some unique properties. Microorganisms can synthesize biopolymers belonging to the classes (i) to (vi), and among them are numerous polymers which are used by industry for technical applications in medicine, pharmacy and agriculture, as packaging materials and in many other areas. Biotechnological production of these polymers is, at present, mostly achieved by the fermentation of microorganisms in stirred-tank bioreactors, and the biopolymers can be obtained as extracellular or intracellular compounds. Alternatively, biopolymers can be also produced by enzymatic *in vitro* processes. Biopolymers belonging to the classes (vii) and (viii) are mainly synthesized by eukaryotic organisms and most abundantly by plants.

Principles of Biopolymer Synthesis: Location of Biosynthesis

All biopolymers are synthesized by enzymatic processes in the cytoplasm, in the various compartments or organelles of cells, at the cytoplasmic membrane or at cell wall components, at the surface of cells or even extracellularly. Synthesis of a biopolymer may be initiated in one part of a cell and may be continued in another part as it occurs, for example, during the synthesis of complex cell wall constituents in bacteria. There are also numerous examples of the transport of polymers from one compartment of a cell to another as it may be required, for example, for some proteins in the mitochondria, and chloroplasts in eukaryotic organisms. Polymers can be also excreted from cells into the environment. This occurs, for

Prof. Dr. Alexander Steinbüchel was born in Lüneburg (FRG) 47 years ago. His interest in microbiology began with undergraduate studies at the University of Göttingen and was extended through his research work in the field of enzyme fermentation undertaken for his Diploma and PhD theses with Prof. Schlegel at the same university. After a year at the Rockerfeller University (New York) in the department of Prof. Christian DeDuve, he returned to the University of Götingen where he completed his habilitation in 1991. He spent one month as a Visiting Professor at the University of Buenos Aires and since September 1994 has held the Chair of Full Professor of Microbiology and has been the Director of the Institut für Mikrobiologie at the Westfälische Wilhelms-Universität Münster. His current areas of interest are the physiology, biochemistry and genetics of (i) metabolism and biotechnological production of polyhydroxyalkanotes, (ii) biosynthesis and biotechnological production of polyamides, (iii) degradation of natural and chemosynthetic polymers, (iv) microbial degradation of actoin, (v) regulation of fermentative metabolism in aerobic bacteria and (vi) microbial transformation of flavour compunds. A highlight of his career is the award of the "Philip Morris Forschungspreis" received with H. G. Schlegel and G. Gottschalk for the production of biodegradable thermoplastic polyesters from renewable resources. He has over 200 publications to his name and is an active member of the editorial boards of many journals including Macromoecular Bioscience. Prof. Dr. Alexander Steinbüchel is married and has 3 children.

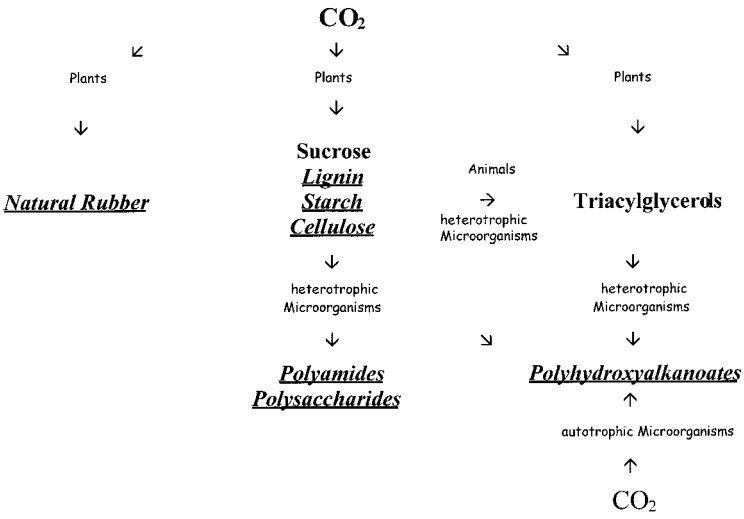

Figure 1. Major renewable carbon sources available from plants and autotrophic microorganisms.

example, in enzyme proteins which hydrolyze polymeric nutrients or lipids. Furthermore, polymers such as plasmids or other parts of the genomes can be taken up by cells in processes referred to as transformation or conjugation.[1, 3]

Principles of Biopolymer Synthesis: Template-dependent and -independent Processes

Biopolymers are either synthesized by template-dependant or template-independant enzymatic processes. This difference has significant consequences for the structure and the molecular weight of the polymers. Nucleic acids are synthesized with desoxyribonucleic acid (DNA) or ribonucleic acid (RNA) as a template, whereas messenger RNA (mRNA) is the template for the synthesis of proteins by ribosomes (Table 1). Nucleic acids and proteins exhibit a complex primary structure in which the constituents are, from a purely chemical point of view, randomly distributed. A template guarantees that the complex primary structure of these two classes of polymers is highly conserved. Another consequence of the template-dependant biosynthesis of polymers is that the resulting polymers are monodisperse, i.e., consist of individual molecules all possessing exactly the same molecular weight. In contrast, poly(amino acids) and members of the classes (iii) to (viii), which are synthesized by template-independant enzymatic processes (Table 1), are polydisperse, i.e., the individual molecules of a particular biopolymer species do not exhibit a uniform molecular weight. If the molecular weight of the latter is described, usually the weight average molecular weight and the molecular weight distribution indicated by the polydispersity index are analyzed, in the same way as for polymers obtained from synthetic processes.[4]

Principles of Polymer Biosynthesis: Substrates of the Polymerizing Enzymes

Most biopolymers are not simply synthesized by the direct polymerization of their building blocks. For example, glycogen is not synthesized by the polymerization of glucose, but from ADP-glucose or UDP-glucose.[5] Table 1 lists in a general way those intermediates of the metabolism that are used by the polymerizing enzyme systems as substrates. If no activated or energy-rich compound is used for the polymerization reaction, the reaction may be driven by the hydrolysis of ATP as, for example, during biosynthesis of poly(γ-D-glutamate)[6] or another energy-rich cosubstrate. Well known exceptions from these two rules are the synthesis of, for example, dextran and some other glucans or fructans.[7] A special situation occurs during the synthesis of lignin which relies on the enzymatic formation of radicals derived from phenylpropane units and spontaneous chemical reaction.[8]

Biopolymers and Renewable Resources

In addition, biopolymers can be obtained from agriculture or from biotechnological processes and are therefore, in principle, available from renewable resources. Figure 1 provides an overview on the major products which are available from plantations of crops and trees (i.e., from agriculture and forestry) and which are used for non-food applications. With the exception of triacylglycerols and sucrose, these products are mainly polymers such as starch, cellulose, lignin and natural rubber. By far the most important producers are plants. Autotrophic microorganisms may be also candidates for the synthesis of some of these biopolymers; however, as yet, only weak perspectives for a biotechnological production of biopo-

lymers have been outlined, and only for polyhydroxyalkanoates employing chemolithoautotrophic[9] and photoautotrophic[10] microorganisms. In contrast, heterotrophic microorganisms are considered as suitable producers of several biopolymers such as PHAs, polysaccharides and polyamides. Many biopolymers possess rather complex chemical structures and compositions and are therefore not available from chemical synthesis. Furthermore, biopolymers, like almost all products of living matter, are generally biodegradable, whereas this is not a general feature of synthetic polymers.[11,12] These few aspects indicate reasons for the growing interest of industry to produce and use biopolymers for a steadily increasing number of applications.[13] Only few biopolymers have been commercialized so far; this is mainly due to the production costs, which are often much higher than for synthetic polymers that have been established in the market during the last decades. However, this situation may change for some biopolymers in the future, when biotechnological production processes have been further optimized and if our knowledge of the material properties and processing of biopolymers has increased.

Isolation and Production of Biopolymers

There are different ways to produce biopolymers in order to make them available for interesting technical applications: (i) Many biopolymers occur abundantly in nature and are isolated from plants and algae which grow in natural environments or are cultivated on plantations. Cellulose and starch are isolated from several agricultural crops and trees such as *Zea maize* or *Pinus silvestris*, respectively. Natural rubber is isolated from plantantations of the rubber tree *Hevea brasiliensis*, and agar and alginates are isolated from red algae belonging to the genus *Gelidium*[14] or from various brown algae also referred to as seaweeds,[15] respectively. (ii) Few biopolymers are isolated from extremely scarce natural sources. An example of such an exception is hyaluronic acid which is extracted from the umbilical cords of new born children.[16] (iii) *In vitro* synthesis of biopolymers with isolated enzymes in cell-free systems offers another possibility to produce biopolymers. One example is the application of the heat-stable DNA polymerases in the polymerase chain reaction (PCR) to produce monodisperse defined DNA molecules.[17] Another example is dextran, which can be produced on a technical scale with isolated dextran sucrase.[18] (iv) Fermentative production of biopolymers is used by industry to obtain, for example, polysaccharides such as xanthan and dextran by employing the bacteria *Xanthomonas campestris*[19] or *Leuconostoc mesenteroides*,[7] respectively. Microbial cellulose obtained by fermentation of *Acetobacter xylinum* seems to offer some advantages for medical applications over cellulose which has been isolated from plants.[5] The homopolyester poly(3-hydroxybutyrate), poly(3HB), and the copolyester poly[(3-hydroxybutyrate)-*co*-(3-hydroxyvalerate)], poly(3HB-*co*-3HV), which were available under the trade name "Biopol" from ZENECA and Monsanto, have been also produced on an industrial scale by employing *Ralstonia eutropha*.[20] The aforementioned examples are not based on genetically modified bacteria; however, genetically engineered bacteria can also be employed for the production of biopolymers. (v) Finally, there are many efforts to generate transgenic plants for the production of certain biopolymers. Examples are plants producing Biopol[21] or amylose and amylopectin.[22]

II Impacts of Advanced Technologies and Methodologies on Biopolymers

There are many reasons for scientists from academia and industry to be interested in biopolymers. Firstly, during the investigation of biological aspects of biopolymers and their metabolism scientists will still find many "white spots" since our knowledge about the physiology, biochemistry and molecular genetics of biopolymers is often scarce. Secondly, many biopolymers have unique properties, and practically all of them are biodegradable in contrast to most synthetic polymers. Thirdly, since chemical synthesis is neither possible nor economically feasible, even if the structure of a biopolymer seems to be not too complex, organisms are often the only source for these polymers which can therefore be obtained at lower costs or higher purity. They are either isolated directly from higher organisms as they occur in nature or on plantations of agricultural crops or trees or they are biotechnologically produced by the fermentation of microorganisms. Therefore, they are directly or indirectly available from CO_2 or renewable resources.

During the last decade basic and applied research have revealed much knowledge on the biochemical and molecular basis of the enzymatic processes for polymer synthesis in microorganisms. The combination of detailed physiological studies, utilization of the overwhelming information provided by the numerous genome sequencing projects, application of recombinant DNA technology, engineering of metabolic pathways or enzymes, and molecular breeding techniques applied to plants, provided new perspectives to produce technically interesting biopolymers by novel or significantly improved biotechnological processes or by agriculture. Geneticists and molecular biologists have developed many powerful methods for *in vitro* and *in vivo* modification of DNA.[23] In addition, efficient methods are available for the transfer of DNA and expression of heterologous genes which are applicable to many organisms. Metabolic engineering has become an important and powerful approach in biotechnology[24] (Table 2). Successful metabolic engineering

Table 2. Tools for successful and powerful metabolic engineering.

- A large box of sophisticated *in vitro* and *in vivo* molecular methods for modification of DNA and for transfer of recombinant DNA into other organisms
- Detailed knowledge on physiology and metabolism
- Diversity of microorganisms
- Genome sequencing projects provide an overwhelming amount of data

requires, however, a detailed knowledge of the physiology and metabolism of the respective organism to which these technologies are applied. It also requires knowledge and experience to utilize the large diversity of microorganisms for example as sources for useful genes.[25,26] Scientists knowing only *E. coli* or being only perfect in DNA sequencing will have problems, as will scientists who have never worked on a larger scale than that of an agar plate or a tiny reaction tube.

III Intracellular versus Extracellular Production of Biopolymers

The biotechnological production of biopolymers may occur intracellularly or extracellularly. This causes several severe consequences regarding the limitations of the production and downstream processes to obtain the biopolymers in a purified state (Figure 2).

PHAs,[27] cyanophycin,[28] glycogen,[29] starch[30] and polyphosphate[31] are examples of biopolymers which are accumulated in the cytoplasm of cells. The availibility of space in the cytoplasm therefore limits the amount of polymer that can be produced by a cell. This is particularly relevant for fermentative production processes mostly employing microorganisms. Therefore, the yield per volume is limited/determined by the cell density and the fraction of the biopolymer in the biomass. Another general consequence is that more or less tedious processes must follow the production of the biomass containing the biopolymer to disintegrate the cells or tissues and to release the biopolymer from the cells. Furthermore, other cell constituents will be released concommitantly with the biopolymer and must then be separated.

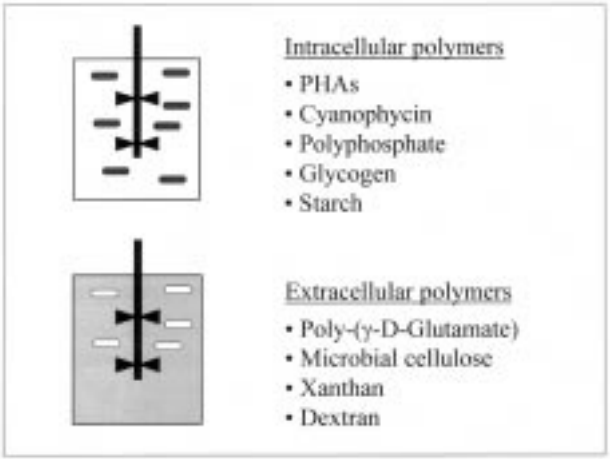

Figure 2. Examples of biopolymers which are produced intracellularly and extracellularly and a demonstration of the "volume" problem.

Poly(γ-D-glutamate)[32] and many polysaccharides, such as alginates,[15] dextran,[7] xanthan,[19] and microbial cellulose[5] are examples of biopolymers which occur outside the cells, either as a result of extracellular synthesis or of excretion by the cells. For these biopolymers, the volume of the cytoplasm is not a limiting factor, and, in principle, the entire volume of the bioreactor (instead of only that of the cytoplasm) would be available to deposit the desired biopolymer. Furthermore, breakage of cells or tissues is not required and separation of the biopolymer from the other biomass is not very complex. However, biotechnological processes can merely take advantage of these features since the presence of these mostly water-soluble biopolymers in the medium usually causes a high viscosity in the medium, resulting in rheological problems during the fermentation process. Unfortunately, hydrophobic water-insoluble biopolymers of biotechnological interest that occur extracellularly are not known. Therefore, in practice, the amount of biopolymer produced per volume by extracellular processes is usually lower than can be obtained by intracellular processes.

Other strategies and the use of cell-free production processes, may take advantage of the features of extracellular processes. One strategy is to apply *in vitro* synthesis of biopolymers employing isolated enzymes. Another strategy is to produce the constituents of polymers as monomers by fermentative processes and to polymerize these components subsequently by solely chemical processes. Both these strategies have already entered reality and many different examples of scale have been demonstrated (i.e., not only at the laboratory scale but also at the technical scale). Polylactide acid, for example, will be produced on a large scale by such a combined biotechnological and chemical approach.[33] The third and most difficult and pretentious strategy would be to convert an intracellular process into an extracellular process by metabolic and genetic engineering of the cells or organisms. This could be done by, for example, extending the capability of a cell to excrete a polymer which is synthesized intracellularly. However, to the best knowledge of the author, this has not been demonstrated, yet.

IV Occurrence of Natural Polyesters

Three different types of naturally occurring organic polyesters and one inorganic polyester are known (Figure 3). These are polyoxyesters occurring in the prokaryotic bacteria and archaea, polymalic acid occurring in

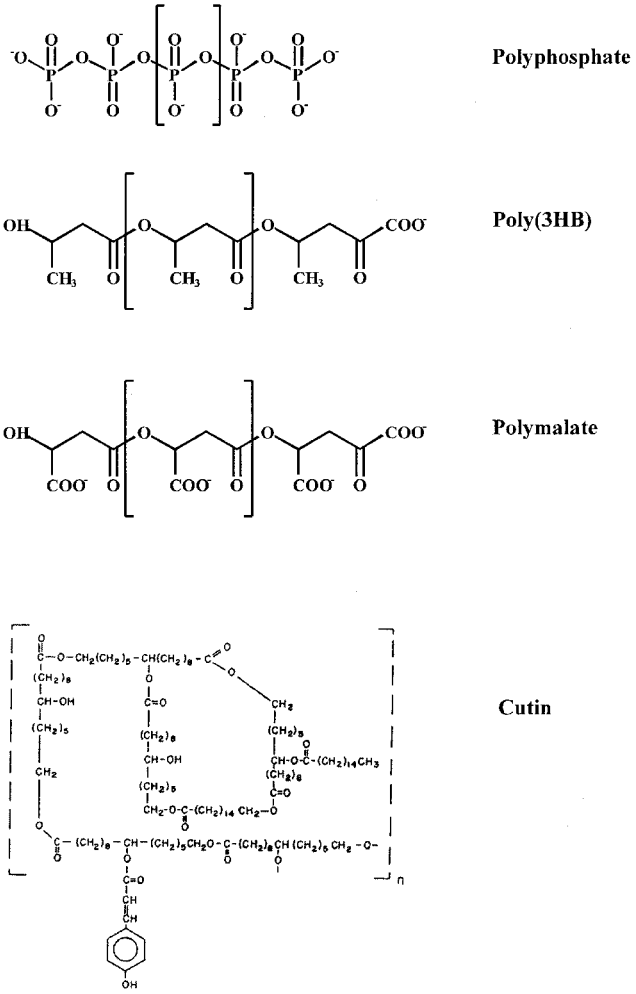

Figure 3. Structural formula of organic and inorganic polyesters.

eukaryotic microorganisms, and cutin and suberin occurring in plants. In addition, the inorganic polyester polyphosphate occurs in organisms of all the kingdoms. As far as it is known, all these types of polyesters are synthesized by different mechanisms and are catalyzed by enzymes exhibiting quite different characteristics.

Bacterial Polyoxyesters

An overwhelming number of different polyhydroxyalkanoates (PHAs), comprising approximately 150 different hydroxyalkanoic acids as constituents, has been isolated from bacteria during the last 20 years.[34] Accumulation of PHAs in the bacterial cell usually occurs if a carbon source is provided in excess, and if at least one other nutrient, which is essential for growth, has been depleted, i.e., if growth is imbalanced.[27,35] These water insoluble polyesters accumulate in the cytoplasm and are deposited as cytoplasmic inclusions which are referred to as PHA granules.[36] They serve as storage compounds for energy and carbon. PHAs are synthesized by diverting either central intermediates of the carbon metabolism or derivatives from precursor substrates, which are provided as carbon source for the growth of the bacteria, to hydroxyacyl-CoA thioesters.[37] The thioesters are then polymerized by PHA synthases[38,39] that are bound to the surface of PHA granules together with other proteins.[36,40,41]

Polymalic Acid

This is the only water-soluble polyester occurring in living matter.[42] This anionic homopolyester is synthesized by *Physarum polycephalum*[43,44] and a few lower eukaryotic microorganism such as *Penicillium cyclopium*[45] and *Aureobasidium pullulans.*[46,47] So far, no bacteria have been identified which synthesize polymalic acid. The biosynthesis of this polyester is, as yet, only poorly understood, and polymerization seems to occur by a mechanism different from that of the bacterial polyoxoesters.[48] This biopolyester is mentioned here only for completness and will not be further considered in this review.

Cutin and Suberin

Cutin and suberin are complex and crosslinked copolyesters which are only synthesized by plants and occur as structural components of the cuticle covering the aerial parts of the plants.[49] Cutin consists of a large variety of different monohydroxy acids, dihydroxy acids, tri- and pentahydroxy acids, epoxy and oxi acids and also dicarboxylic acids; predominant constituents are, for example, 10,16-dihydroxy C_{16} acid, 18-hydroxy-9,10 epoxy C_{18} acid and 9,10,18-trihydroxy C_{18} acid. Suberin consists of aromatic domains derived from cinnamic acid and aliphatic polyester domains with predominantly long-chain fatty acids, fatty alcohols, ω-hydroxy fatty acids and dicarboxylic acids as its main constituents. The biosynthesis of these two polyesters, in particular the polymerization reaction, is not yet fully understood.[50] Since this review focuses on bacterial PHAs, cutin and suberin will be not further considered in this review.

Polyphosphates

In this inorganic polyester, orthophosphate residues are linked by phosphoanhydrid bonds. Polyphosphate is synthesized by prokaryotes, eukaryotic microorganisms and even higher eukaryotic organisms including mammals, presumably as a storage compound for energy and phosphate.[31] In the cells, it occurs mostly as insoluble cytoplasmic inclusions as complexes with divalent cations. The biosynthesis and degradation of this polyester is well understood.[31]

V Applied Aspects of Polyhydroxyalkanoic Acids

Properties of PHAs

All PHAs share some properties which recommend them for some applications and make them interesting to industry.[51–54] (i) They are thermoplastic and/or elastomeric compounds which can be processed with apparatus used by the plastic manufacturing industry. In addition, they are (ii) insoluble in water, (iii) exhibit a rather high degree of polymerization ranging from 10^5 to almost 10^7 Da, and (iv) they are enantiomerically pure chemicals consisting, in general, only of the *R*-stereoisomer. They are (v) non-toxic and (vi) biocompatible and (vii) exhibit piezoelectric properties as revealed (at least) for poly(3HB) and poly(3HB-*co*-3HV). (viii) Several PHAs can be obtained from CO_2 or renewable resources. Finally, (ix) all PHAs are biodegradable; they are hydrolyzed by extracellular PHA depolymerases, and the cleavage products are subsequently utilized as sources of carbon and energy by many bacteria and fungi.[55] PHAs consisting of ω-hydroxyalkanoic acids are, in addition, also hydrolyzed nonspecifically by lipases and esterases.[56, 57] In addition, PHA accumulating bacteria usually possesses intracellular PHA depolymerases, which mobilize the storage compound if no other carbon source is available to the cells.[58]

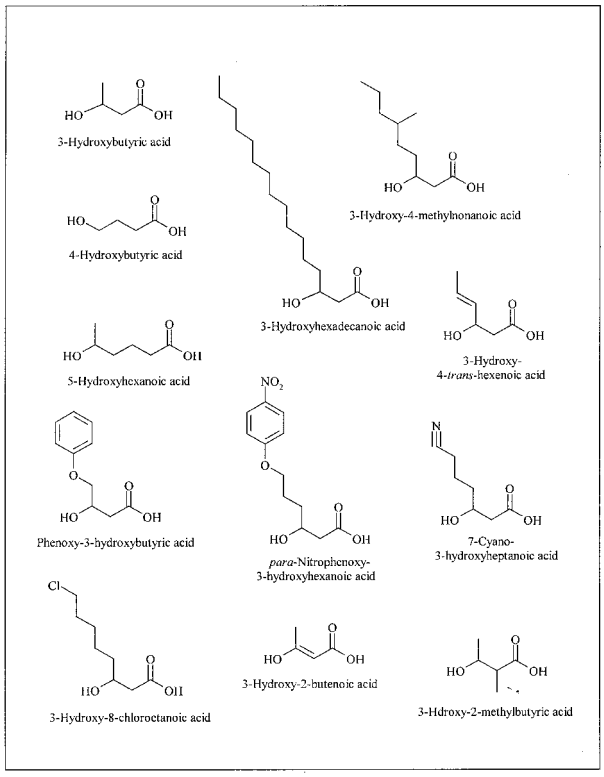

Figure 4. Some examples of constituents of biosynthetic PHAs.

Uses and Applications of PHAs

Commercial production of PHAs is so far only possible by fermentative biotechnological processes. Several PHAs, such as, in particular, the homopolyester poly(3HB), the copolyester poly(3HB-*co*-3HV) and PHAs consisting of 3-hydroxyoctanoate, 3-hydroxydecanoate and a few other medium-chain-length 3-hydroxyalkanoates, poly($3HA_{MCL}$), have been manufactured into various materials, and applications in various areas have been revealed. Whereas the use of poly(3HB) and poly(3HB-*co*-3HV) as biodegradable bioplastics was established some time ago,[51] other applications such as the manufacturing of latex paints,[59] and specifically medical applications including retard materials[60] and use as scaffolding material for tissue engineering,[61] are currently under development. Isolated and purified PHAs can be used as such or in combination with other materials such as starch, cellulose fibers, glass fibers or synthetic plastics to obtain compounded materials.[62] Also, from the purified material new polymers can be obtained by transesterification in the melt in the presence of synthetic polyesters,[63] by chemical modification of the side chain[64] or by crosslinking through energized irradiation and chemical reagents.[65] Another interesting application of PHAs is the use of these polymers as a source to obtain enantiomeric pure hydroxyalkanoic acids upon chemical or enzymatic cleavage for the synthesis of chemicals.[66] There have also been applications for bacterial cell mass containing a high fraction of PHAs as binders for other fibrous materials.[67]

VI *In Vivo* Biosynthesis of PHAs and PHA Synthases

As already mentioned, approximately 150 different hydroxyalkanoic acids are at present known as constituents of these bacterial storage polyesters.[34] Some examples of these constituents demonstrating the variabilities of the structures and sizes of hydroxyalkanoic acids detected in PHAs are shown in Figure 4. PHAs are synthesized by diverting intermediates of the carbon metabolism to hydroxyacyl-CoA thioesters. The latter are then polymerized by PHA synthases which are bound to the surface of PHA granules together with other proteins. However, most of these PHAs are only obtained if precursor substrates, which are structurally more or less related to the constituents to be incorporated into PHAs, are provided as a carbon source for the bacteria, because central intermediates can not be diverted to the respective hydroxyacyl-CoA thioesters. Only a few PHAs can therefore be obtained from simple carbon sources which are available in large amounts from agriculture, such as carbohydrates and fatty acids, or from the CO_2 of the atmosphere.

Table 3. Three classes of PHA synthases. The features of the three classes which distinguishes PHA synthases with respect to size, subunit composition and substrate specificities are shown. Well-studied PHA synthases of each class are mentioned as well as the distribution of these PHA synthase classes in the groups of microorganisms.

Class	Subunits	Occurrence	Substrates
I		***Ralstonia eutropha*** + most other bacteria except those listed below	$3HA_{SCL}$-CoA [$\sim C_3$–C_5] also: $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA
II		***Pseudomonas oleovorans*** + all *Pseudomonas* sp. belonging to rRNA homology group I	$3HA_{MCL}$-CoA [$\sim \geq C_5$]
III		***Chromatium vinosum*** ***Thiocapsa pfennigii*** + other sulfur purple bacteria + all cyanobacteria	$3HA_{SCL}$-CoA [$\sim C_3 - C_5$] ($3HA_{MCL}$-CoA [$\sim C_4 - C_8$] also: 4HA-CoA, 5HA-CoA)

Since the cloning of the PHA operon of the Gram-negative bacterium *Ralstonia eutropha* approximately 12 years ago, more than 50 PHA synthase structural genes, and also many other genes related to PHA biosynthesis from various other bacteria, have been cloned, and many of them have been analyzed at a molecular level.[38, 39] These genes comprise the structural genes for (i) PHA synthases, (ii) granule-associated proteins and (iii) enzymes, which catalyze the formation of hydroxyacyl-CoA thioesters, as well as probably also genes for (iv) proteins that have a regulatory function.

PHA synthases can be classified into three different classes according to their structure and substrate range (Table 3). Class I comprises PHA synthases consisting of only one type of subunit with molecular weights ranging from approximately 60 to 65 kDa in most cases. Another characteristic of class-I PHA synthases is that predominantly hydroxyalkanoic acid with three, four or five carbon atoms are incorporated into PHAs. This class is represented by the PHA synthase of *R. eutropha* and occurs in all other poly(3HB) accumulating bacteria[68–85] accept the sulfur purple bacteria and the cyanobacteria. Class II comprises PHA synthases also consisting of only one type of subunit and with a similar molecular weight range; however, the substrate specificities of PHA synthases belonging to this class is different. These PHA synthases incorporate medium-chain-length 3-hydroxyalkanoic acids into PHAs. The chain length characteristically comprises 6 to 14 carbons with a high freedom for the structure of the *R*-pendant group. This class is represented by the enzyme of *Pseudomonas oleovorans* and seems to occur exclusively in pseudomonads *sensu strictu*.[86–92] Class III comprises PHA synthases consisting of two different types of subunits each with a molecular weight of approximately 40 kDa. One subunit exhibits significant homologies to the class-I and class-II PHA synthases with all highly conserved amino acids also present, but lacking extended regions of the amino terminal and the carboxy terminal regions. PHA synthases of this class occur obviously in all cyanobacteria[93–95] and in all sulfur purple bacteria[96–99] but not in the non-sulfur purple bacteria[100–103] investigated so far. The class-III PHA synthases of *Chromatium vinosum* and *Thiocapsa pfennigii* have been studied in greatest detail. One reason for this was the unusual structure of this PHA synthase. The other reason was the unusual substrate range. For the PHA synthase of *T. pfennigii*, it was demonstrated that the enzyme was able to synthesize copolyesters of 3HB and various medium-chain-length hydroxyalkanoic acids, even those with the hydroxyl group not at the 3′ position.[98, 99, 104]

VII Why Search for Novel PHAs?

As mentioned above, approximately 150 different hydroxyalkanoic acids have been already detected as constitu-

Table 4. How to get novel routes to specific PHAs

- Isolation of new wild type strains of bacteria
- Generation of mutants
- Use of precursor substrates
- Application of inhibitors
- *In vitro* metabolic engineering
- *In vitro* metabolic engineering
- Expression of PHA synthase in a different physiological background
- Expression of HA-CoA biosynthesis pathways in organisms expressing PHA synthase
- Molecular engineering of PHA synthases or enzymes providing substrates for PHA synthases
- A combination of strategies listed above

ents of bacterial PHAs. However, there is still ongoing interest in the search for novel PHAs. This means the search for PHAs with novel constituents (new HA), a new combination of already known constituents (for example a copolyester [-X-Y-X-Y-] instead of a blend of two homopolyesters [–X-X-X-X-] plus [–Y-Y-Y-Y-]) or a new order of constituents (for example a block copolyester [–X-X-X-X-Y-Y-Y-Y-] instead of a random copolyester [-X-Y-X-Y-X-Y-X-Y]). In addition, novel types of biopolymers can be obtained applying a polymerizing enzyme system, for example, to unusual substrates, as was shown with the detection of polythioesters when mercaptoalkanoic acids were used as a precursor carbon source for the PHA-accumulating *R. eutropha*.[2, 105] Such studies are not only to the satisfaction of microbiologists and biochemists, which try to reveal the true substrate range of these fascinatingly unspecific PHA synthases, but may have also far-reaching impacts on the provision of novel biopolymers applicable to the manufacturing of polymeric materials exhibiting novel and improved physical and material properties.

VIII Strategies to Obtain Novel PHA Biosynthesis Pathways and Improved Production Strains

There are several possibile means to obtain bacteria exhibiting routes to novel PHAs or novel routes to already known PHAs or organisms with improved capabilities to produce PHAs. They are listed in Table 4 and the physiological basis of these strategies, and the possible effect of the measures are shown in Figure 5, which also indicates possible targets for screening and metabolic engineering. It is, of course, also possible to apply a combination of two or even more of these approaches.

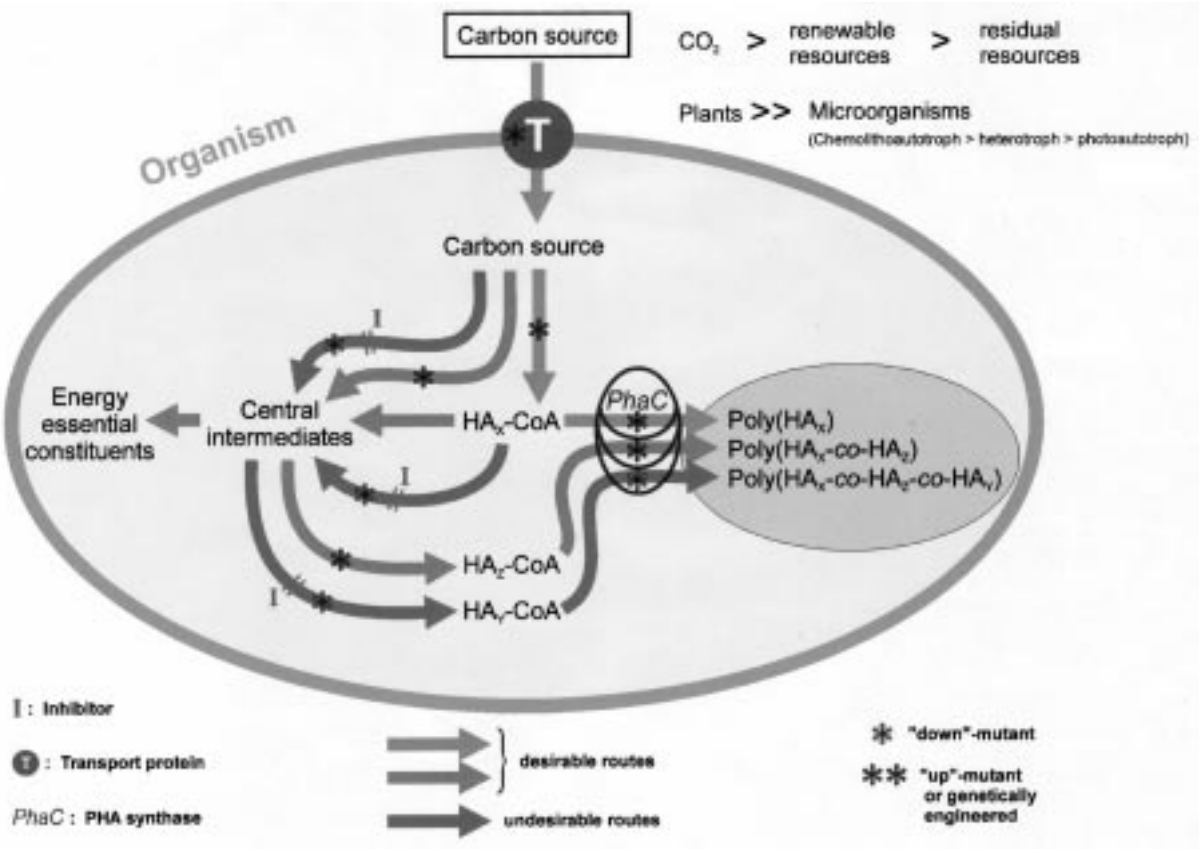

Figure 5. Targets for screening and metabolic engineering to obtain suitable organisms or strains for production of PHA.

Isolation of New Wild-Type Strains of Bacteria

Many Bacteria accumulate poly(3HB), and most of them, like *R. eutropha*, synthesize this polyester from acetyl-CoA in a three-step pathway via acetoacetyl-CoA and (*R*)-3-hydroxybutyryl-CoA catalyzed by the enzymes *β*-ketothiolase, a pyridine nucleotide-dependant acetoacetyl-CoA reductase and the PHA synthase.[106] Microbiologists isolated and identified several bacteria after extended screening processes which were able to synthesize PHAs consisting of constituents different from 3HB from simple, structurally non-related carbon sources such as glucose. The two most important examples comprise the copolyester poly(3HB-*co*-3HV) and PHAs consisting of medium-chain-length 3-hydroxyalkanoic acids ($3HA_{MCL}$).

Almost all pseudomonads *sensu strictu* synthesize poly($3HA_{MCL}$), not only when they are cultivated on alkanes such as octane[107] or organic acids such as octanoic acid,[108] but also from glucose and many other carbon sources.[109, 110] *P. oleovorans* has been well studied with respect to the formation of PHA consisting mainly of (*R*)-3-hydroxyoctanoate from octane or octanoic acid. Octane is oxidized to octanoic acid by oxygenases and dehydrogenases, and the latter is then activated to octanoyl-CoA and further oxidized via *β*-oxidation. Intermediates of the *β*-oxidation cycle are then polymerized to the polyester. *P. oleovorans* is one of the few pseudomonads *sensu strictu* unable to synthesize poly($3HA_{MCL}$) from unrelated substrates although the genes encoding this pathway are present (see below).[111] Most other pseudomonads, such as *P. putida* and *P. aeruginosa*, posses this pathway and accumulate during cultivation on gluconate as carbon source a copolyester consisting of (*R*)-3-hydroxydecanoate as main constituent and (*R*)-3-hydroxydodecanoate and (*R*)-3-hydroxyoctanoate as minor constituents.[109, 110] Cultivation experiments employing labelled carbon sources and inhibitors provided evidence that the fatty acid *de novo* synthesis pathway is the major pathway for the provision of the 3-hydroxyacyl moieties in *P. putida*.[112] An acyltransferase was recently isolated and the gene cloned from *P. putida*, which transfers the hydroxyacylmoiety from (*R*)-3-hydroxydecanoyl-acyl carrier protein to coenzyme A thus forming (*R*)-3-hydroxydecanoyl-CoA, which is a substrate of the two PHA synthases in this bacterium.[92, 115] This key enzyme links fatty acid *de novo* synthesis and poly($3HA_{MCL}$) in *P. putida* and is also present in *P. aeruginosa*[114] and other pseudomonads.[115] A highly homologous gene was also identified in the genome of *P. oleovorans,*[111] however, in this bacterium, the gene is either silent or inactive, since this bacterium cannot accumulate poly($3HA_{MCL}$) from, for example, gluconate.

Poly(3HB-*co*-3HV) with very high contents of 3HV are synthesized by various species belonging to the Gram-positive genera *Nocardia* or *Rhodococcus*.[116] Pyruvate, which is formed during the degradation of glucose via the 2-keto-3-deoxy-6-phosphogluconate pathway in these bacteria, is probably carboxylated to oxaloacetate and subsequently converted to succinyl-CoA by the reverse citric acid cycle which is then converted via methylmalonyl-CoA to propionyl-CoA, as revealed by analysis of *N. corallina* and *R. ruber*[117–119] Propionyl-CoA is then condensed with acetyl-CoA yielding 3-ketovaleryl-CoA which is reduced to (*R*)-3-hydroxyvaleryl-CoA and together with (*R*)-3-hydroxybutyryl-CoA polymerized to poly(3HB-*co*-3HV). This is a very good example of the endogenous generation of propionyl-CoA. Otherwise, precursor substrates such as propionic acid, valeric acid or other fatty acids with an odd number of carbon atoms,[120] α,ω-alkanediols with an odd number of carbon atoms,[121] levulinic acid[122, 123] or certain amino acids such as valine or isoleucine[124–127] must be used as a carbon source during the cultivation of the bacteria to obtain poly(3HB-*co*-3HV). Actually, propionic acid is used in addition to glucose in the Biopol process for the production of poly(3HB-*co*-3HV) with *R. eutropha*.[128–129]

Other interesting, newly isolated bacteria are *Pseudomonas* sp. 61-3 and *Aeromonas caviae* which are able to accumulate copolyesters of 3HB and $3HA_{MCL}$. Whereas *Pseudomonas* sp. 61-3 accumulated a copolyester of 3HB and $3HA_{MCL}$ from sugars as well as from fatty acids of medium chain length,[130] *A. caviae* accumulated a copolyester of 3HB and 3-hydroxyhexanoate during cultivation on fatty acids of medium chain length.[131]

Generation of Mutants

Furthermore, from these and many other bacteria, mutants were isolated which exhibited advanced features for the accumulation of PHAs. The literature provides many examples of such mutants. It would break the scope and length of this review to mention all these mutants in detail and it is therefore recommended to refer to previous reviews.[27, 37, 39, 132–134]

Use of Precursor Substrates

Rather frequently and abundantly, precursor substrates were used as carbon sources to cultivate various bacteria under conditions which permitted the synthesis and accumulation of PHAs. Most of the hydroxyalkanoic acids known to be constituents of PHAs were identified upon such experiments. Despite the detection of so many different PHAs, the use of precursor substrates is still another a tool to obtain novel, hitherto unknown PHAs. One recent example are polythioesters, consisting, for example, of 3-mercaptopropionic acid or 3-mercaptobutyric acid.[2, 105] This entirely new type of biopolymer is

obtained in *R. eutropha* due to the broad unspecificity of the PHA synthase.

The description of hitherto unknown, sulfur-containing polythioesters was the first report on a natural polymer containing sulfur in its backbone.[2, 105] *R. eutropha* synthesized a copolymer of 3-hydroxybutyrate and 3-mercaptopropionate, poly(3HB-*co*-3MP), when 3-mercaptopropionic acid or 3,3′-thiodipropionic acid was provided as a carbon source, in addition to fructose or gluconic acid, under nitrogen-limited growth conditions. The peculiarity of this polymer was the occurrence of thioester linkages derived from the thiol groups of 3MP and the carboxyl groups of 3MP or 3HB, respectively, which occurred in addition to the common oxoester bonds of PHAs. Depending on the cultivation conditions and the feeding regime, poly(3HB-*co*-3MP) contributed up to 19% of the cellular dry weight, with a molar fraction of 3MP of up to 43%. The chemical structure of poly(3HB-*co*-3MP) was confirmed by gas chromatography/mass spectrometry, infrared spectroscopy, ^{1}H- and ^{13}C nuclear magnetic resonance spectroscopy, and elemental sulfur analysis. Therefore, poly(3HB-*co*-3MP) can be designated as the first representative of an eighth class of biopolymers, namely polythioesters. Another hitherto unknown copolymer that contains sulfur in its backbone, is a polythioester covalently linking 3-hydroxybutyrate and 3-mercaptobutyrate, poly(3HB-*co*-3MB). It was also synthesized by *R. eutropha*, when 3-mercaptobutyric acid was fed as a carbon source in addition to gluconate. The total polymer yield contributed to up to 31% of the cellular dry weight. Elemental sulfur analysis of poly(3HB-*co*-3MB) revealed a total sulfur content of 11.65% (w.-%), thus the molar fraction of 3MB was calculated as 33.24 mol-%. The molecular structure of this novel polymer was also confirmed by gas chromatography/mass spectrometry, infrared spectroscopy, and ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy.

Application of Inhibitors

The flux of intermediates may be influenced and directed by the use of inhibitors. This approach will be helpful for basic studies since it may reveal information about the flux of metabolites and by which pathways and enzymes substrates for the PHA synthase are provided. However, the data have to be carefully analyzed because inhibitors do interfere in most cases, with more than one particular enzyme if they are not very specific. Therefore, artifacts may occur, leading to a false interpretation of the data. Considering the biotechnological production of PHAs, the inhibitors will probably not be applicable because of the extra costs and because the inhibitor will have to be completely removed from the polyester during purification if the polyester is to be used for food applications, or for medical or pharmaceutical applications. In addition, whereas inhibitors may be more easily applied during submers cultivation of microorganisms from a technical point of view, they are difficult to apply to the production of PHAs in transgenic plants. Therefore, for large scale production of PHAs, it might be better to use strains in which the effect of the inhibitor is simulated by a mutation or by other approaches in order to direct the flow of metabolites in the desired direction. The application of various inhibitors was quite useful during the analysis of poly($3HA_{MCL}$) and the establishment of poly($3HA_{MCL}$) biosynthesis in a recombinant strain of *E. coli* (see section IX).

In Vivo Metabolic Engineering

The availibility of the key enzyme PHA synthase and of enzymes involved in the biosynthesis of hydroxyacyl coenzyme A thioesters and detailed knowledge on the biochemistry of these enzymes as well as of additional structural and regulatory proteins will allow engineering of the metabolism to obtain new PHA biosynthesis pathways. In addition, it will be essential to establish functional active PHA biosynthesis pathways in organisms that are, themselves, not able to accumulate PHAs but are considered as more suitable for commercial production of PHAs either by fermentative processes or by cultivation of transgenic crops. To achieve this, knowledge about putative regulatory events effecting the activity of the enzymes or the expression of these enzymes or other proteins might also be important. *In vivo* metabolic engineering is of particular interest if pathways are engineered which mediate between central intermediates of the metabolism on one side and the hydroxyacyl coenzyme A thioesters as substrates of PHA synthases on the other side. This is because such studies could yield recombinant bacteria or transgenic plants which are able to produce a wider range of PHAs from simple and cheap carbon sources or CO_2, respectively. The current possibilities and achievements regarding production of the various PHAs by fermentation of wild-type and recombinant bacteria were recently extensively reviewed.[134] Of particular interest is the engineering of pathways for the synthesis of coenzyme A thioesters of hydroxyalkanoic acids which differ from 3HB-CoA in order to obtain organisms suitable for the production of a wider range of different PHAs from cheap and abundantly available carbon sources. Some examples for the *in vivo* engineering of metabolic links between the fatty acid metabolism and poly($3HA_{MCL}$) will follow (see section IX).

In Vitro Metabolic Engineering

In vitro synthesis of biopolymers means that the polymerizing enzyme acts in a cell-free system and catalyzes the formation of the polymer from its substrate(s). Various

assays are available for measuring PHA synthase activity.[135] For other enzymes, like an alcohol dehydrogenase, it is quite normal to measure the NADH-dependant reduction of acetaldehyde by, for example, a photometric assay. In contrast, for polymer-synthesizing enzymes, the assay of its activity is often considered to be something special, most probably due to the fact that the enzymes are often more complex and require more cofactors and, in some cases, also addition of a template and a primer. If polymer biosynthesis is done with a highly active and more or less purified enzyme preparation, the biopolymer can be obtained in such quantities, that it can be isolated and subsequently characterized with respect to its composition. Depending on the amount of the polymer, which can be prepared, even some physical and/or material properties may be analyzed. Such studies will not only reveal important data and knowledge on mechanistic aspects and other characteristics of the enzyme, but may also allow the synthesis of novel biopolymers which would not be available from the enzyme acting *in vivo*. The reasons for this are the extension to substrates and primers, which can not be synthesized in a cell but are available from chemical synthesis, and the possibility to switch from one substrate to another within a short period of time, thus allowing the synthesis of, for example, block copolymers.

If the polymer synthesizing enzyme is combined with other enzymes, short pathways can be engineered. It may be a pathway as it also exists in cells *in vivo* or it may be a pathway which is completely artifical and is not occurring in cells and has therefore to be referred to as a none-natural pathway. In both cases enzymes from different organisms may be combined. Later, some examples for the *in vitro* metabolic engineering will be provided (see section X).

Molecular Expression of PHA Synthases or Enzymes Providing Substrates for PHA Synthases

As already mentioned, approximately 50 PHA synthase structural genes and many genes encoding enzymes catalyzing the formation of hydroxyacyl-coenzyme A thioesters have been cloned from different microorganisms and characterized at a molecular level.[38, 39] Many of these genes have been heterologously expressed at least once in a foreign host, probably most often in one of the PHA-negative mutants of *R. eutropha* (for example mutant PHB_4^- [134]) and in *E. coli*. The most detailed and comprehensive studies have been done with the PHA synthase operon of *R. eutropha*, which consists of the structural genes of *β*-ketothiolase (*phaA*), NADPH-dependent acetoacetyl-CoA reductase (*phaB*) and PHA synthase (*phaC*) occurring in the order *phaCAB*. This operon, or at least *phaC*, was successfully expressed in many different bacteria, in *Saccharomyces cerevisiae*, in animal cells and in plant cells and established, in most cases, functional PHA biosynthesis. Expression in *E. coli* and plants will most probably have the greatest impact with respect to the production of PHAs in the future. Therefore, two separate sections of this review article are devoted to this aspect (see sections XI and XII).

Table 5. Establishment of PHA biosynthesis in non-PHA accumulating organisms

1. Genetic transfer of the PHA synthase gene
2. Expression of active PHA synthase
3. Appropriate provision of the substrate for the PHA synthase
   (i) Provision from "precursor" carbon sourves
   (ii) Provision from central intermediates
   - The desired hydroxyalcyl-coenzyme A thioester must be synthesized in the cell (in the right tissue and compartment) and accumulated to a certain extent
   - The withdrawal of intermediates for the synthesis of this hydroxyacyl-coenzyme A thioester must not impair the normal function of the cell

IX Metabolic Engineering of Poly($3HA_{MCL}$) Biosynthesis Pathways

Metabolic engineering of PHA biosynthesis pathways requires the establishment of a functional pathway that allows the conversion of the carbon source to an intermediate which is further routed to the desired PHA synthase substrate and finally polymerized. This may mean the establishment of an entire pathway hitherto not present in the particular organism or only modifications of a few or even a single step in the sequence of a pathway.

It should be noted that the genetic transfer of a PHA synthase gene is only a first step (Table 5). Further measures must ensure that the PHA synthase is expressed in a functionally active form. Finally, and this is the most important aspect and may be the most difficult to achieve, the substrate for the PHA synthase must be available in the cytoplasm or in the compartment of the cell in which the PHA synthase is expressed. The best PHA synthase and a formidable activity of this enzyme will be useless if the enzyme has no substrate. The substrate may be provided from a precursor substrate, which is more or less directly converted into the desired hydroxyacyl coenzyme A thioester, or, better, from a central intermediate. This requires that a pathway for this conversion exists in the cell or, if eukaryotic microorganisms are considered, in the right compartment of the cell, and, if higher eukaryotic organisms are considered, also in the right tissue of the respective organism. Furthermore, the withdrawal of intermediates for PHA biosynthesis must not impair the normal functions of the cell; otherwise the cell, and most likely the entire organism, may be negatively affected, and growth may be retarded. The latter was often a serious problem during the establishment of PHA biosynthesis in transgenic plants (see section XII).

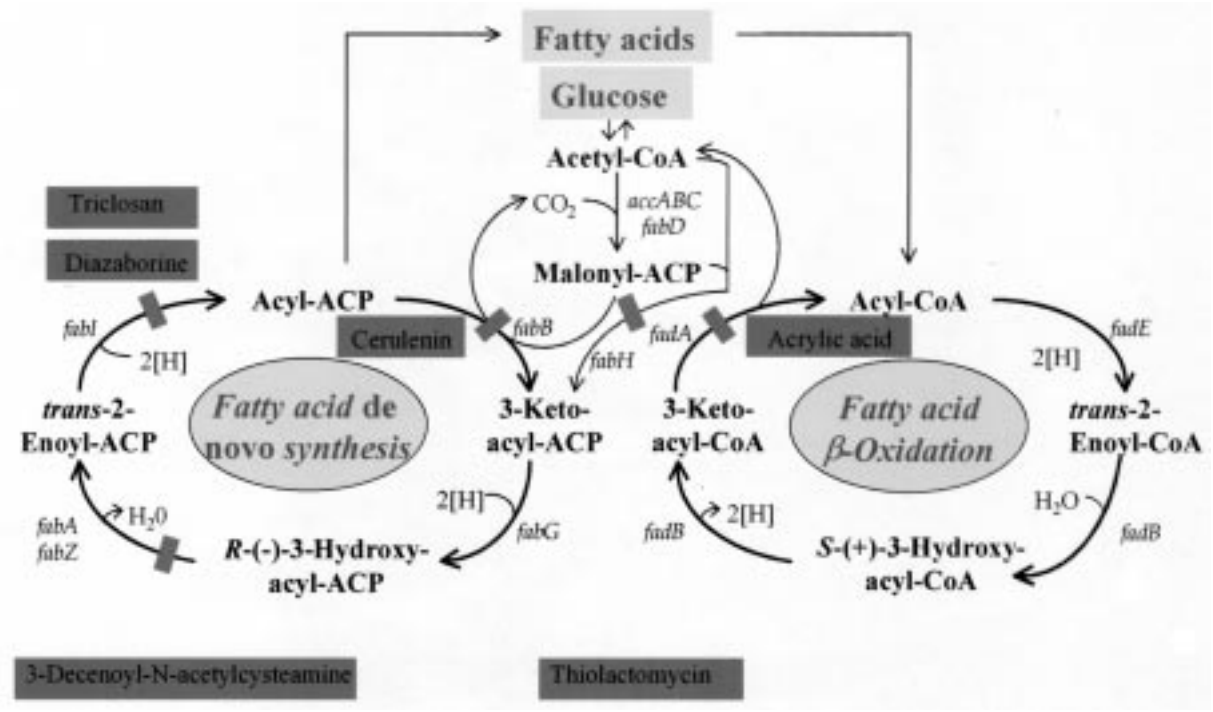

Figure 6. Inhibitors of fatty acid metabolism.

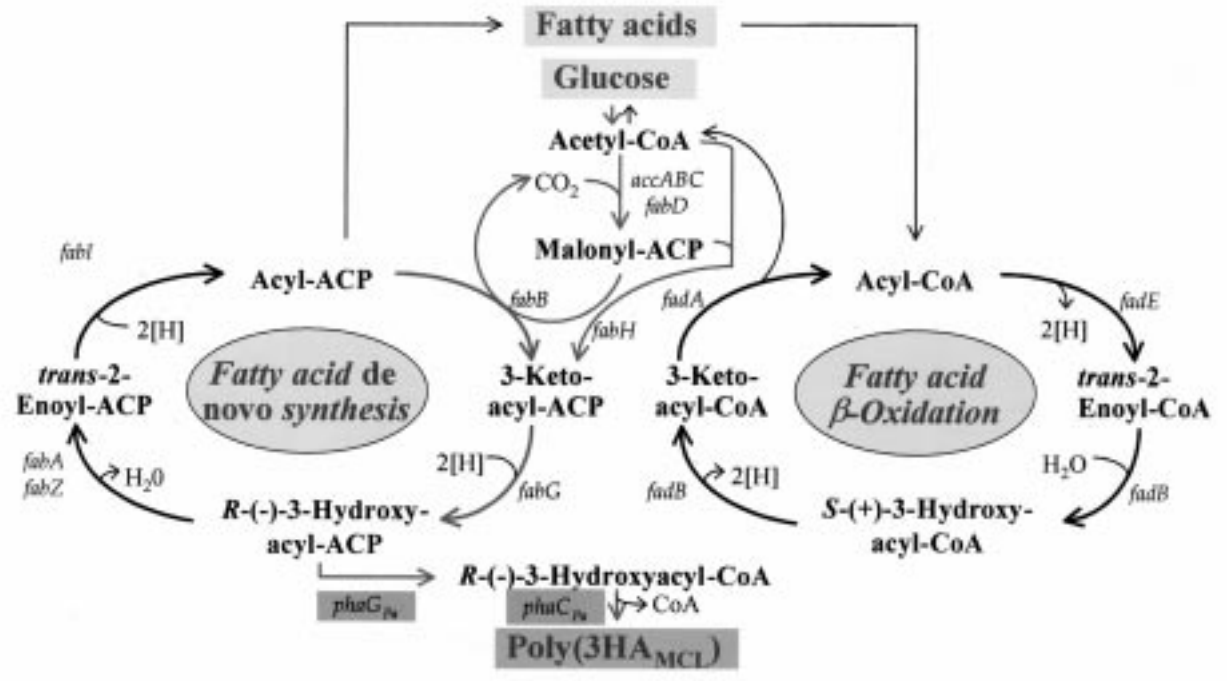

Figure 7. Metabolic link between fatty acid *de novo* biosynthesis and poly($3HA_{MCL}$) biosynthesis.

The biosynthesis of poly($3HA_{MCL}$) in pseudomonads has been investigated in depth and the application of inhibitors was found to be quite useful. Figure 6 shows the reactions of the fatty acid *de novo* synthesis pathway and of the fatty acid β-oxidation pathway for the conversion of glucose or fatty acids, respectively; in addition, the enzymes catalyzing the various reactions are indicated with reference to the respective genes encoding these enzymes. This figure also shows six chemicals which more or less specifically inhibit one or the other reaction. Whereas acrylic acid[137]

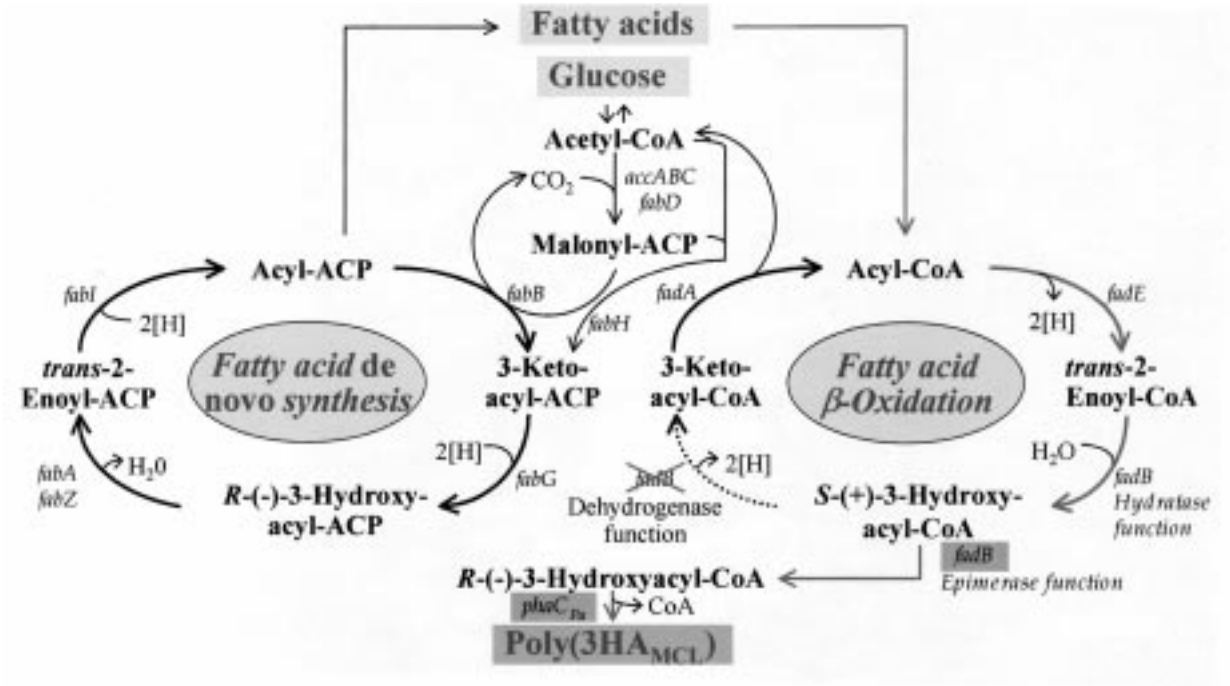

Figure 8. Metabolic link between fatty acid β-oxidation and poly(3HA$_{MCL}$) biosynthesis in recombinant *E. coli* exhibiting a *fadB* mutation.

inhibits the β-ketothiolase of the β-oxidation pathway, thiolactomycin,[138] 3-decenoyl-*N*-acetylcysteamine,[139] triclosan,[140] diazaborine[141] and cerulenin[142] inhibit enzymes of the fatty acid *de novo* synthesis pathway.

During study of poly(3HA$_{MCL}$) biosynthesis in *P. putida* from glucose, the application of the inhibitor cerulenin revealed that fatty acid *de novo* synthesis plays a major role providing approximately 90% of the PHA constituents. Provision of substrates by chain elongation through β-ketothiolases or from cell lipids through the β-oxidation pathway, the latter as revealed by application of the inhibitor acrylic acid, are significant, but contribute to only a minor fraction of the constituents.[112] Mutants were isolated which grew normally on any carbon source tested and which still synthesized poly(3HA$_{MCL}$) from fatty acids but not from gluconate or glucose.[113] The phenotype of these mutants indicated a defect in the enzyme converting intermediates of the fatty acid *de novo* synthesis pathway into a substrate of the PHA synthases in *P. putida*. The enzyme was purified, and biochemical studies revealed that the enzyme catalyses the transfer of (*R*)-3-hydroxydecanoyl moieties from the acyl carrier protein to coenzyme A (Figure 7); this reaction was dependant on magnesium ions. It was referred to as a 3-hydroxyacyl-acyl carrier protein : coenzyme A transferase, and the respective gene, which complemented the mutant, was referred to as *phaG*.[113] *PhaG* has been also identified and cloned from *P. aeruginosa*,[114] *P. oleovorans*[111] and *Pseudomonas* sp. 61–3[143] and seems to be present also in other pseudomonads *sensu strictu* such as *Pseudomonas* sp. DSM1645, *P. mendocina* and *P. citronellolis* as revealed by hybridization experiments.[111] Therefore, a highly conserved transacylase provides the metabolic link between fatty acid *de novo* synthesis and PHA synthase in the *Pseudomonas* species *sensu strictu*.

In subsequent experiments, *phaG* from *P. putida* was transferred to and expressed in *P. oleovorans* which is able to synthesize poly(3HA$_{MCL}$) from alkanes or fatty acids, but not from gluconate or fructose, and exhibits, therefore, a similar phenotype as the *phaG* mutants of *P. putida* (see above). Furthermore, *phaG* from *P. aeruginosa* was, together with the PHA synthase gene from *P. aeruginosa*, transferred to the non-PHA accumulating bacterium *P. fragi,* and both were heterologously expressed in this host (Figure 7).[144] When the recombinant strains were cultivated with gluconate as a carbon source under conditions, which permitted the accumulation of PHAs, the cells accumulated poly(3HA$_{MCL}$) contributing approximately 50% of the cell dry matter in the case of *P. oleovorans* and 10% in the case of *P. fragi*, respectively, whereas in the parent strain or in a recombinant strain harboring only the vector, PHAs were not detected. These two studies demonstrated that the cloned transacylase is functionally active and can be used to establish poly(3HA$_{MCL}$) synthesis in other bacteria.

Regarding the metabolism of poly(3HA$_{MCL}$) in pseudomonads, another open question was the metabolic link between fatty acid β-oxidation and PHA synthase. Although a 3-hydroxyacyl coenzyme A intermediate occurs, it represents the wrong stereoisomer. In principle,

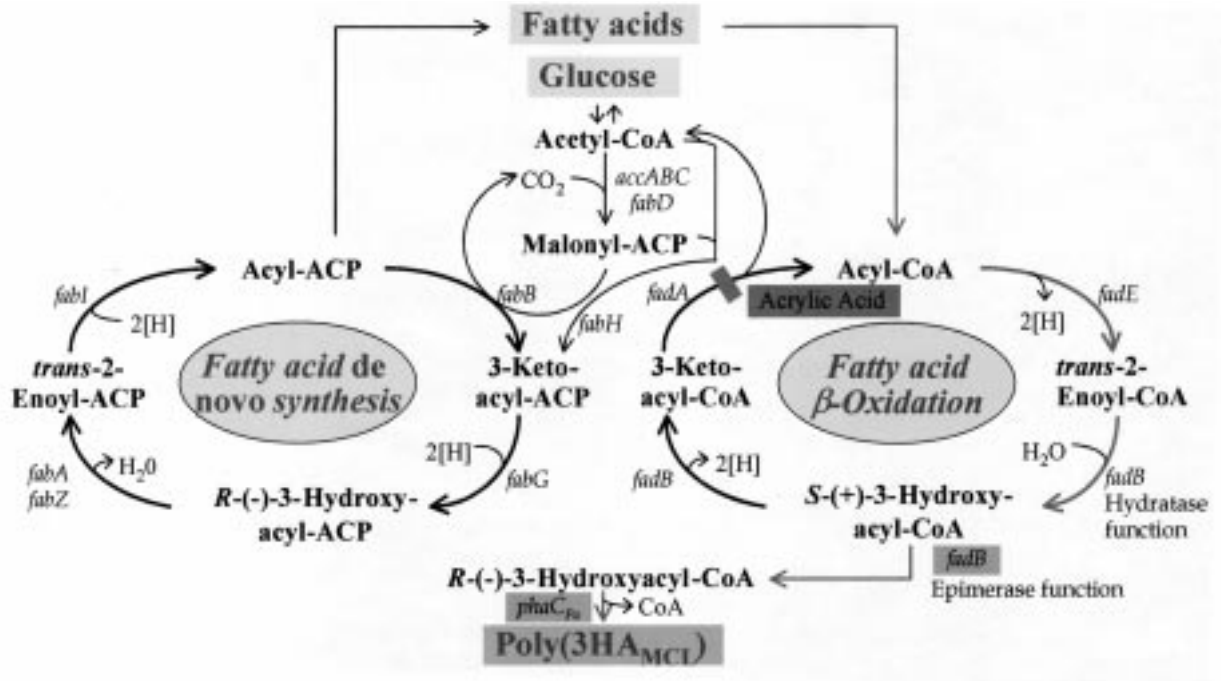

Figure 9. Metabolic link between fatty acid β-oxidation and poly(3HA$_{MCL}$) biosynthesis in recombinant *E. coli* in which β-oxidation is inhibited by acrylic acid.

three enzymes may putatively provide this metabolic link: (i) a 3-ketoacyl-CoA reductase reducing 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoA, (ii) an epimerase converting the (*S*)-isomer of 3-hydroxyacyl-CoA into the (*R*)-isomer and (iii) a hydratase converting *trans*-2-enoyl-CoA into (*R*)-3-hydroxyacyl-CoA. During our attempts to establish poly(3HA$_{MCL}$) biosynthesis in *E. coli*, the recombinant strains expressed PHA synthase but accumulated only trace amounts of PHAs if cultivated on decanoic acid or various other fatty acids (Table 6). Only if the *fadB* mutant LS1298 of *E. coli* was used, were PHAs accumulated at a significant level, contributing 20–25% of the cell dry matter[145] (Table 6). In mutant LS1298, the dehydrogenase function is defective, whereas the hydratase and also the epimerase functions of FadB remain active[146] (Figure 8). Obviously, (*S*)-3-hydroxyacyl-CoA accumulates in the cytoplasm of the mutant cells to a level which is sufficiently high to be effectively converted into the (*R*)-stereoisomer and subsequently also into PHA. Instead of *phaC1*, *phaC2* of *P. aeruginosa* was also used, and very similar results were obtained.[147] This is a good example of the efficient engineering of the metabolic fluxes of intermediates of a degradative pathway towards a new product which is not normally synthsized by the cells. This system allowed the synthesis of quite a large range of different PHAs, depending on the fatty acid which was used as carbon source. The length of the incorporated 3-hydroxyalkanoic acids reflected the length of the carbon chain of the fatty acid provided as carbon source.[145, 147]

Table 6. Influence of acrylic acid on accumulation of PHA$_{MCL}$ in recombinant strains of *E. coli* harboring plasmid pBHR71 from decanoic acid as carbon source.

Strain of *E. coli*	Property	PHA$_{MCL}$ content of the cells [wt.-% of CDW]	
		–Acrylic acid	+Acrylic acid
LS1298	*fadB*	21.1	19.5
K12	–	2.8	25.4
JM109	–	1.1	29.9
XL1-Blue	–	0.9	27.9
RS3097	*fadR*41	<0.2	45.7

Cultivations were done in LB medium containing 4 g · l^{-1} decanoic acid, 1 mM IPTG and ±0.2 g · l^{-1} acrylic acid.
Abbreviations: CDW, cell dry weight; PHA$_{MCL}$, polyhydroxyalkanoates consisting of medium-chain-length 3-hydroxyalkanoates. The content of this table was taken from ref.[148]

A different system for the production of poly(3HA$_{MCL}$) by *E. coli*, allowing a larger flexibility, was established employing the inhibitor acrylic acid[148] (Figure 9). When recombinant strains of *E. coli* expressing either *phaC1* or *phaC2* from *P. aeruginosa* were cultivated on fatty acids such as decanoic acid, in the presence of low concentrations of this inhibitor, PHAs were accumulated by any strain of *E. coli* investigated (Table 6). Several strains even accumulated significantly higher amounts of PHA than the mutant LS1298 in the absence of acrylic acid. Most PHA was accumulated by a *fadR* regulatory mutant, in which poly(3HA$_{MCL}$) contributed approximately 45%

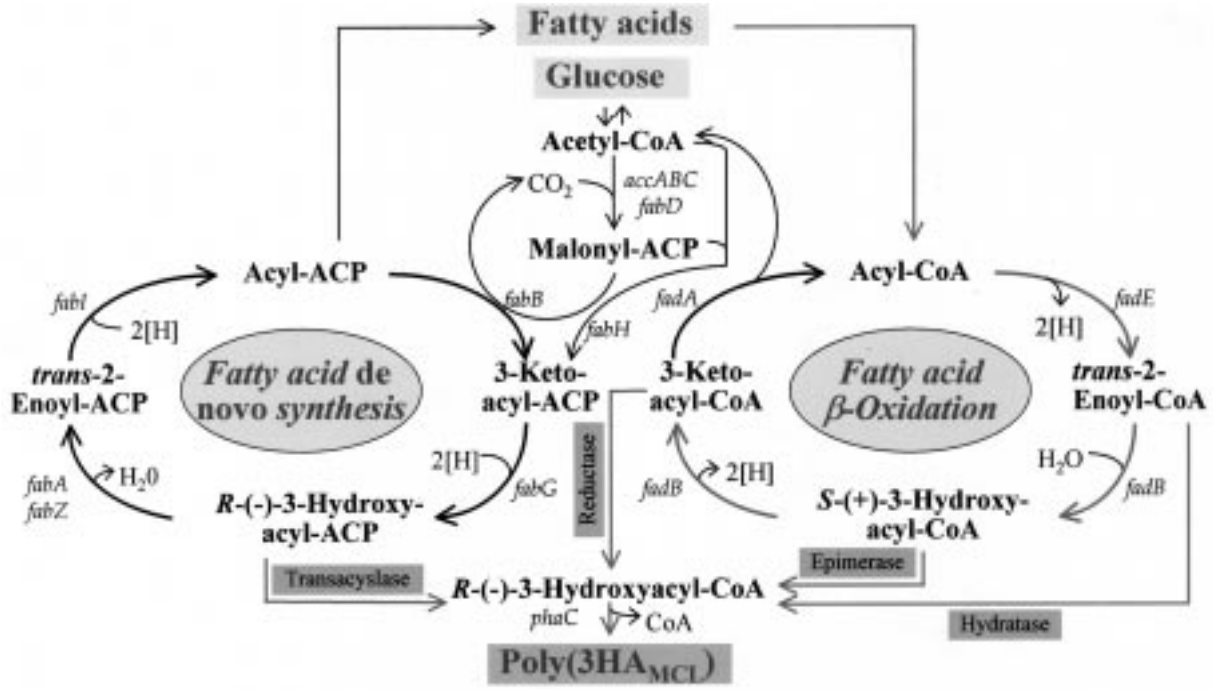

Figure 10. Metabolic links between fatty acid metabolism and poly(3HA$_{MCL}$) biosynthesis – an overview.

of the cellular dry matter (Table 6). Obviously, the addition of acrylic acid and the inhibition of the β-ketothiolase have a similar effect as a defective FadB dehydrogenase function with respect to the accumulation of (*S*)-3-hydroxyacyl-CoA and the routing of intermediates of the β-oxidation cycle towards poly(3HA$_{MCL}$) biosynthesis and accumulation (Figure 9).

Further studies by other laboratories established the relevance of the reductase and the hydratase for PHA biosynthesis. In *Aeromonas caviae*, a (*R*)-specific enoyl-CoA hydratase is responsible for the conversion of trans-2-enoyl-CoA to (*R*)-3-hydroxyacyl-CoA during the cultivation of this bacterium on, for example, hexanoate.[78] Two (*R*)-specific enoyl-CoA hydratase genes were also identified in *P. aeruginosa*.[149] Studies in recombinant strains of *E. coli* provided evidence that FabG of fatty acid *de novo* synthesis, which is a 3-ketoacyl-ACP reductase, is non-specific and also exhibits activity with the corresponding CoA-thioesters.[150] On the other hand, it was shown that recombinant strains of *E. coli* could also use their own β-ketothiolase (FadA) in combination with the acetoacetyl-CoA reductase (PhaB) of *R. eutropha* for the conversion of fatty acids into poly(3HA$_{MCL}$).[151] Figure 10 summarizes the possible metabolic links between the fatty acid *de novo* synthesis pathway and poly(3-HA$_{MCL}$) biosynthesis with the transacylase on one side, and between the fatty acid β-oxidation pathway and poly(3HA$_{MCL}$) biosynthesis with the reductase, the epimerase and the hydratase on the other side.

As an additional note, acrylic acid and cerulenin were also applied to investigate the competition of the pathways for poly(3HB-*co*-3HV) and triacylglycerol (TAG) biosynthesis in species of the genus *Rhodococcus*.[152] Cerulenin completely inhibited the accumulation of TAGs from glucose or gluconate but not from hexadecane and caused an increase, not only of the content of poly(3HB-*co*-3HV), but also of the molar fraction of 3HB in the copolyester in *R. ruber*. On the other hand, acrylic acid completely inhibited the accumulation of TAGs from valerate and caused a twofold increase of the PHA content which consisted exclusively of 3HV.[152] These studies nicely demonstrated how the two different pathways compete strongly for the intermediates as substrates.

X *In Vitro* Biosynthesis of PHAs

Six different systems have been described in the literature for the *in vitro* biosynthesis of PHAs employing purified PHA synthases and other enzymes (Figure 11). All PHA synthases employed in these studies were isolated from cells of recombinant strains of *E. coli* since efficient purification procedures for PHA synthases from the original hosts have not been elaborated. Four systems used a combination of at least two enzymes which originated from different organisms and therefore represent engineered pathways.

The PHA synthases of *R. eutropha*[153, 154] and *Chromatium vinosum*[155] have been used to synthesize poly(3HB)

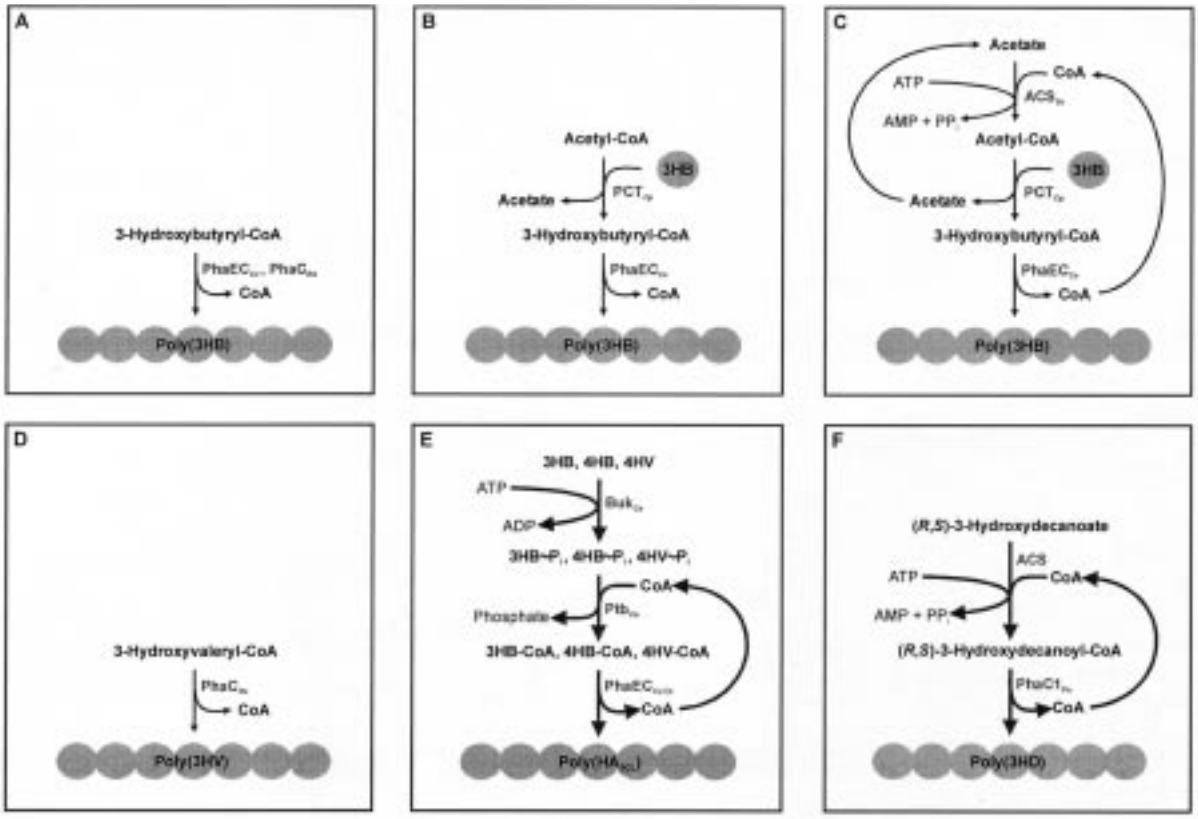

Figure 11. *In vitro* PHA biosynthesis systems. This figure summarizes the different *in vitro* PHA biosynthesis systems that have been described in the literature. A: One-step system for synthesis of poly(3HB) employing the PHA synthase of *Ralstonia eutropha*[153, 154] or *Chromatium vinosum*,[157] B: Two-step system for synthesis of poly(3HB) employing the propionyl-CoA transferase of *Clostridium propionicum* plus the PHA synthase of *C. vinosum*,[155] C: three-step system for synthesis of poly(3HB) employing the acetyl-CoA synthetase of *Saccharomyces cerevisiae,* propionyl-CoA transferase of *C. propionicum* plus the PHA synthase of *C. vinosum,*[155] D: one-step system for synthesis of poly(3HV) employing the PHA synthase of *R. eutropha,*[161] E: three-step system for synthesis of PHA_{SCL} consisting of 3HB, 4HB and/or 4HV employing the butyrate kinase of *C. acetobutylicum*, phosphotransbutyrylase of *C. acetobutylicum* plus the PHA synthases of *C. vinosum or Thiocapsa pfennigii,*[159] F: two-step system for synthesis of poly(3HD) employing an acyl-CoA synthetase of *Pseudomonas* sp. plus the PHA synthase of *P. aeruginosa.*[162] Abbreviations: ACS, Acyl-CoA synthetase of *Pseudomonas* sp.; ACS_{Sc}, acetyl-CoA synthetase of *S. cerevisiae*; Buk_{Ca}, butyrate kinase of *C. acetobutylicum*; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate, 3HD, 3-hydroxydecanoate; 4HB, 4-hydroxybutyrate; 4HV, 4-hydroxyvalerate; HA_{SCL}, short-chain-length hydroxyalkanoates; PCT_{Cp}, Propionyl-CoA transferase of *C. propionicum*; $PhaC_{Pa}$, PHA synthase of *P. aeruginosa*; $PhaC_{Re}$; PHA synthase of *R. eutropha*; $PhaEC_{Cv}$, PHA synthase of *C. vinosum*; $PhaEC_{Tp}$, PHA synthase of *T. pfennigii*; Ptb_{Ca}, Phosphotransbutyrylase of *C. acetobutylicum.*

in one-enzyme systems from 3-hydroxybutyryl-CoA (Figure 11 a). It was shown that granules similar to those occurring in the cytoplasm were formed, and the size of the granules could be modulated by the addition of a phasin, which is one of the other structural proteins *in vivo* bound to the surface of PHA granules. There are two problems with such one-enzyme systems: Firstly, the costs are extraordinarily high due to the use of 3-hydroxybutyryl-CoA. To synthesize one gram of poly(3HB), the cost of the substrate alone is approximately 500.000 US$. Secondly, coenzyme A, which is released from the substrate in stoichiometric amounts, inhibits the PHA synthases, and, therefore, the reaction does not proceed well or to a large extent. Therefore, multiple-enzyme systems were developed to use cheaper substrates and to recycle the cofactors, in particular coenzyme A. Employing a two-enzyme system using purified PHA synthase of *C. vinosum* and propionyl-CoA transferase from *Clostridium propionicum,*[156] acetyl-CoA could be converted into poly(3HB) (Figure 11 b).[157] This system was extended to a three-enzyme system by adding commercially available purified acetyl-CoA synthetase from yeast (Figure 11c).[157] In this system, acetate was used as a substrate and was subsequently converted into poly(3HB). Coenzyme A could be recycled, allowing the use of only catalytic instead of stoichiometric amounts of this expensive coenzyme. Since the concentration of coenzyme A remained low in this system, no inhibition

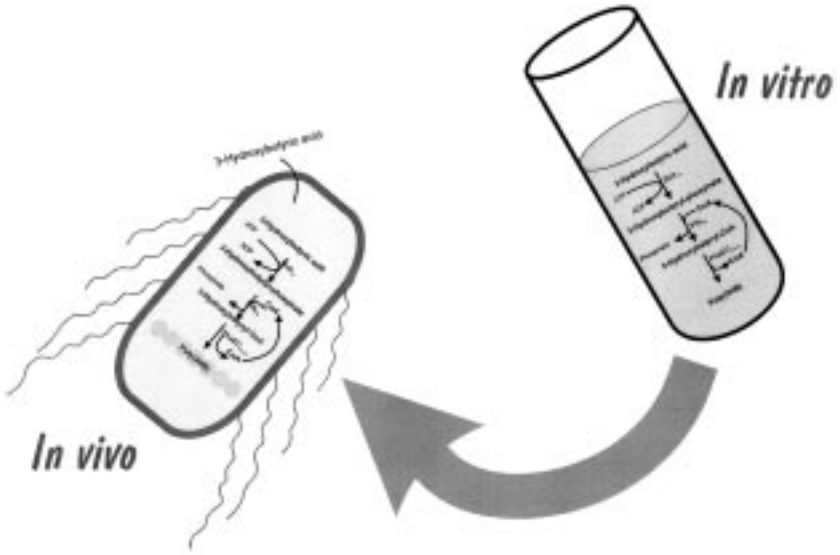

Figure 12. Integration of an *in vitro* engineered poly(3HB) biosynthesis pathway into the metabolism of *E. coli*.

of the PHA synthase could occur. The reaction was driven by the hydrolysis of ATP to AMP and pyrophosphate. The substrate costs for the synthesis of one gramm of poly(3HB) were reduced to approximately 15 US $. These costs are already sufficiently low to allow the synthesis of enough PHAs for the investigation of the physical properties and maybe even for material testing.

Another three-enzyme system allowing synthesis of poly(3HB) from 3-hydroxybutyrate via 3-hydroxylphophate and 3-hydroxybutyryl-CoA with coenzyme A recycling was engineered employing active butyrate kinase (Buk) and phosphotransbutyrylase (Ptb) from *Clostridium acetobutylicum*[158] isolated from recombinant strains of *E. coli*, plus the PHA synthase of *C. vinosum* (Figure 11e).[159] In this system, the reaction was driven by the hydrolysis of ATP to ADP, allowing different strategies for the recycling of ATP and for the polymerization of 3-hydroxybutyryl-CoA. Since the inspection of the substrate specifities of all three enzymes showed that they were also able to use the respective 4-hydroxybutyrate and 4-hydroxyvalerate compounds as substrates, whether this three-enzyme system could be employed to synthesize PHAs consisting of 4HB and 4HV (Figure 11e) was also investigated. The purified enzymes were indeed successfully exploited to synthesize *in vitro* homopolyesters of poly(4HB) or poly(3HV), as well as copolyesters containing various molar fractions of 3HB, 4HB and 4HV, if the respective hydroxyfatty acids were provided as substrates. Even a terpolyester containing all three hydroxyalkanoic acids was synthesized. The highest 4HB content in these copolyesters was 46 mol-%. Therefore, an interesting enzyme system for *in vitro* synthesis of various PHAs was established.

Is it possible to use an *in vitro* engineered PHA biosynthesis pathway for *in vivo* synthesis of polyesters (Figure 12)? To answer this question the three-enzyme pathway shown in Figure 11e was expressed in recombinant strains of *E. coli* with one modification: the *C. vinosum* PHA synthase was replaced by the PHA synthase of *Thiocapsa pfennigii* ($PhaEC_{Tp}$) because the latter exhibited a broader substrate range.[160] The four genes were cloned into plasmid pBR322, and the resulting hybrid plasmid pBPP1 conferred activities of all three enzymes to *E. coli* JM109. When the recombinant strain of *E. coli* was cultivated in M9 mineral salts medium containing glucose and hydroxyfatty acids as carbon sources, the cells accumulated PHAs. Homopolyesters of 3HB, 4HB or 4HV were obtained from each of the corresponding hydroxyfatty acids. Various copolyesters of those hydroxyfatty acids were also obtained when two of these hydroxyfatty acids were fed in equal amounts.[160] For example, cells fed with 3-hydroxybutyric acid and 4-hydroxybutyric acid accumulated a copolyester consisting of 88 mol-% 3HB plus 12 mol-% 4HB and contributing 69% of the cell dry weight. Cells, which were fed with 3-hydroxybutyric acid and 4 hydroxyvaleric acid, accumulated a copolyester consisting of 94 mol-% 3HB plus 6 mol-% 4HV and contributing 64% of the cell dry weight. Finally, cells fed with 3-hydroxybutyric acid, 4-hydroxybutyric acid and 4-hydroxyvaleric acid accumulated a terpolyester consisting of 85 mol-% 3HB, 13 mol-% 4HB plus 2 mol-% 4HV and contributing 68.4% of the cell dry weight. This study

Table 7. The pros and cons of *in vitro* metabolic engineering.

Advantages and prospects	Limitations and problems
• **Detailed biochemical studies** through characterization of the enzyme(s) becomes possible	• **Purified PHA synthase is required**
• **Application of non natural substrates** PHAs with new constituents may be produced	• **Additional enzymes must be available in a purified stage**
• **Rapid shifts of substrates applicable** PHAs with a novel order of constituents may be produced (e.g. blockcopolyesters)	
• **Cell-free system** Production is limited by the volume of the reactor and not by the volume of the cytoplasm	• **All enzymes must be stable**
Increase of PHA produced per volume is possible	• **Coenzymes must be recycled**
Synthesis may be performed in the presence of compounds or under conditions which are inhibiting cells	
No expression required	
Amount of enzymes can be easily varied	
• **Only few components required** downstream processing will be easier	
• **Requires less efforts than *in vivo* metabolic engineering** fast evaluation of the feasibility of a strategy and the functionality of a "designed" pathway	
• **Special applications** *in situ* PHA formation becomes possible	

demonstrated that a PHA biosynthesis pathway engineered *in vitro* employing purified enzymes is functionally active in *E. coli* and can be utilized for *in vivo* synthesis of PHAs.

Other authors[161] demonstrated the *in vitro* biosynthesis of poly(3HV) employing the PHA synthase of *R. eutropha* (Figure 11 d). However, this system suffers from the same disadvantages as the one-enzyme system for poly(3HB) synthesis shown in Figure 11 a. Another type of PHA was obtained *in vitro* by employing purified PHA synthase of *P. aeruginosa* and a commercially available acyl-CoA synthetase from *Pseudomonas* sp. (Figure 11 f). This two-enzyme system synthesized *in vitro* poly(3-hydroxydecanoate) when a racemic mixture of 3-hydroxydecanoate was used as a substrate.[162] This engineered pathway allowed recycling of coenzyme A and was driven by the conversion of ATP to ADP.

Advantages and limitations of *in vitro* synthesis and production processes are listed in Table 7. *In vitro* engineering of pathways may be a useful strategy to evaluate whether the establishment of a particular pathway in a bacterium by *in vivo* metabolic engineering is feasible.

XI Production of PHAs by Recombinant Strains of *E. coli*

As shown in the above sections, *E. coli* has become an interesting organism for the production of PHAs. Recombinant strains of *E. coli*-accumulating PHAs have been repeatedly mentioned in this review. It should be emphasized that *E. coli* is unable to accumulate poly(3HB) or any other PHAs as a storage compound; there are only very small anounts of a lower molecular weight poly(3HB) found mainly in the cytoplasmic membrane.[163] Meanwhile, several quite different PHAs can be obtained from *E. coli* and these are summarized in Table 8. Not only poly(3HB),[68, 164, 165], but also the homopolyesters poly(4HB)[160, 166, 167] and poly(4HV)[160] have been synthesized in *E. coli*. In addition, copolyesters consisting of 3HB plus 3HV,[168, 169] 4HB[160, 166, 167] or 4HV,[160] a terpolyester consisting of 3HB, 4HB plus 4HV[160] and also PHAs consisting of various $3HA_{MCL}$ have been obtained from *E. coli*.[145, 148, 170, 171]

However, this research aimed, not only at the establishment of a PHA biosynthesis pathway, but also at the generation of strains which might be suitable for the commercial production of PHAs. For this, various strains of *E. coli* were obtained which acccumulated high contents of PHAs when cultivated to high cell densities, and, on the basis of these studies, the production costs at an industrial scale were calculated. It was found that such recombinant strains could produce PHAs at much lower cost than, for example, *R. eutropha*.[172–174] However, the costs were still significantly higher than those for conventional and established plastics such as polyethylene.

Table 8. PHAs available from recombinant *Escherichia coli.*

PHA	Reference
Poly(3HB)	[68] [164] [165]
Poly(4HB)	[160] [166] [167]
Poly(4HV)	[160]
Poly(3HB-*co*-3HV)	[168] [169]
Poly(3HB-*co*-4HB)	[160] [166] [167]
Poly(3HB-*co*-4HV)	[160]
Poly(3HB-*co*-4HB-*co*-4HV)	[160]
Poly($3HA_{MCL}$)	[145] [147] [148] [170] [171]

XII Production of PHAs by Transgenic Plants

Soon after the first PHA biosynthesis genes from bacteria were cloned and the functional expression of the *R. eutropha* poly(3HB) pathway in *E. coli* was demonstrated,[68, 164, 165] microbiologists were approached by scientists studying other organisms, in particular by plant geneticists and plant breeders, who all showed an interest in generating recombinant prokaryotes and eukaryotes producing poly(3HB) and other PHAs in a better way than *R. eutropha*. Besides in *E. coli*, the *R. eutropha* poly(3HB) pathway was also expressed in many other bacteria. In parallel, PHA biosynthesis genes became available from many other bacteria and were also expressed in other hosts. Expression of the *R. eutropha* poly(3HB) biosynthesis pathway was subequently also successfully achieved in the yeast *Saccharomyces cerevisiae*[175]and in cells of *Spodoptera frugiperda*[176] and *Trichoplusia ni*.[177]

Plants were also an interesting target for expressing these genes because transgenic plants could produce PHAs directly from CO_2 and solar energy and, at least theoretically, at costs which are comparable to those of other biopolymers already obtained from plants (see Figure 1).[134] In 1992, the biosynthesis of poly(3HB) in transgenic *Arabidopsis thaliana* was reported.[178] Later, these poly(3HB) biosynthesis genes were also expressed in agricultural crops such as *Brassica napus*,[179] *Gossypium hirsutum*,[180] *Nicotiana tabacum*,[181] *Solanum tuberosum*[182] and *Zea mays*.[183] Table 9 lists the requirements on which the successful generation of transgenic plants suitable for the production of PHAs depends. Most of these requirements have already been met, intensive research is still necessary to meet the others.

Table 9. Requirements for transgenic plants producing PHAs.

(i)	Availibility of bacterial PHA biosynthesis genes.
(ii)	Transfer and functional expression of PHA biosynthesis genes in suitable crops.
(iii)	Single rather than multiple insertions of the heterologous genes into the plant genome.
(iv)	Metabolic engineering to provide the PHA biosynthesis enzymes with appropriate intracellular concentrations of substrates.
(v)	Metabolic engineering to divert from central intermediates substrates others than 3-hydroxybutyryl-CoA for PHA biosynthesis.
(vi)	Sufficiently high PHA content (>15% of plant dry matter).
(vii)	No significant depression of plant growth (<20%) due to PHA accumulation.
(viii)	Sufficient high $\overline{M}_w$ of the PHAs (>650000 g · mol^{-1}).
(ix)	Reliable and simple methods for extraction of PHA from the plant tissues and separation of PHA from residual non-PHA biomass.
(x)	Measures to prevent uncontrolled release and distribution of PHA biosynthesis genes in the environment.
(xi)	Acceptance by farmers and consumers.

Table 10. PHAs available from transgenic plants.

PHA	Reference
Poly(3HB)	[178]
Poly(3HB-*co*-3HV)	[184]
Poly($3HA_{MCL}$)	[185]

Meanwhile, not only poly(3HB) can be produced but so can the copolyester poly(3HB-*co*-3HV)[184] and also PHAs consisting of $3HA_{MCL}$[185] (Table 10). To obtain these other two PHAs, the knowledge and experience about the establishment of PHA biosynthesis in *E. coli* and other bacteria was utilized. It will be still a long time before PHAs can be obtained from agriculture, however, the concepts have been proved, and it has been demonstrated that PHAs can be synthesized in transgenic plants. At present, the PHA content of the cells is still low, and depression of plant growth even at relative low PHA contents seems a major problem which has to be overcome. In addition, the isolation of the PHAs from the plant material has to be investigated. In principle, and as outlined recently,[134] the costs of the production of PHAs in transgenic plants could be of the same order of magnitude as the costs of sucrose, lipids and starch, if all the prerequisites are met. Under these circumstances, PHAs from transgenic plants would be significantly cheaper than conventional plastics. The acceptance by farmers and consumers will be, most probably, not such a problem as with other transgenic plant products, since these transgenic plants will be used for non-food applications and since they might help to save fossil resources by providing a substitute for non-biodegradable plastics which are mainly produced from fossil carbon sources, or by providing new materials.

XIII Conclusions and Outlook

Many biopolymers exhibiting interesting physical and material properties are synthesized by microorganisms, and the screening for bacteria and other organisms which are able to produce such biopolymers more efficiently, or which produce novel biopolymers, should be continued and intensified. Furthermore, relatively little is known about the physiological, biochemical and molecular basis of the biosynthesis pathways of many biopolymers. The latter is even often true for some physiologically important and wide-spread biopolymers. Therefore, it is concluded that the potential of living organisms with respect to the provision of interesting biopolymers has been only partially exploited.

In addition, it is concluded that advanced strategies, technologies and methodologies will open new possibilities and perspectives to obtain more suitable organisms

and optimized processes for the production of biopolymers. Genetic engineering, metabolic engineering and the utilization of microbial genoms, as well as the cloning of genes from non-culturable microorganisms, provide the potential for this. One important aim of further investigations must be the reduction of the production costs to make biopolymers available at lower costs which are competitive with those of established synthetic polymers. An almost essential prerequisite for this will be the synthesis of such biopolymers from renewable resources or directly from CO_2. Another aim must be the detection of novel biopolymers, which exhibit features not shown by synthetic polymers and are therefore of extra value. PHAs are just one example, and as outlined in this review, it was shown that intensive research can successfully address these questions and problems.

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