# Chapter 17
# Manipulation of *Ralstonia eutropha* Carbon Storage Pathways to Produce Useful Bio-Based Products

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**Abstract** *Ralstonia eutropha* is a Gram-negative betaproteobacterium found natively in soils that can utilize a wide array of carbon sources for growth, and can store carbon intracellularly in the form of polyhydroxyalkanoate. Many aspects of *R. eutropha* make it a good candidate for use in biotechnological production of polyhydroxyalkanoate and other bio-based, value added compounds. Manipulation of the organism's carbon flux is a cornerstone to success in developing it as a biotechnologically relevant organism. Here, we examine the methods of controlling and adapting the flow of carbon in *R. eutropha* metabolism and the wide range of compounds that can be synthesized as a result. The presence of many different carbon utilization pathways and the custom genetic toolkit for manipulation of those pathways gives *R. eutropha* a versatility that allows it to be a biotechnologically important organism.

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## Abbreviations

| | |
|---|---|
| A | Gas coefficient |
| C/N | Carbon/nitrogen ratio |
| DAPI | 4′,6-Diamidino-2-Phenylindole |
| 3HB | 3-Hydroxybutyrate |
| 4HB | 4-Hydroxybutyrate |
| 3HHx | 3-Hydroxyhexanoate |
| 3H4MV | 3-Hydroxy-4-methyl valerate |
| 3HV | 3-Hydroxyvalerate |
| IR | Infrared |
| MCL | Medium chain length |
| MMBPP | Malaysia/MIT Biotechnology Partnership Programme |
| MTT | 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide |
| PHA | Polyhydroxyalkanoate |
| PhaB | 3-Acetoacetyl-CoA reductase |
| P(HB-*co*-HV) | Poly(hydroxybutyrate-*co*-hydroxyvalerate) |
| P(HB-co-HHx) | Poly(hydroxybutyrate-*co*-hydroxyhexanoate) |
| PhaC | PHA synthase |
| PhaP | Phasin protein |
| PhaZ | PHA depolymerase |
| PHB | Polyhydroxybutyrate |
| PLA | Polylactic acid |
| POME | Palm oil mill effluent |
| q | Specific rate of gas substrate consumption, in kg/(kg·h) |
| RAD16-I::E | Self-assembling peptide with a sequence of arginine alanine and aspartate repeats ending in alanine and glutamate |
| SB RAS | Siberian Branch, Russian Academy of Sciences |
| SCL | Short chain length |
| TCA cycle | Tricarboxylic acid cycle |
| $X_{CO}$ | Volumetric concentration of carbon monoxide in a gas mixture, in % |

## 17.1 Introduction

*Ralstonia* species can accumulate large amounts of polyhydroxyalkanoate (PHA) under unbalanced growth conditions, where protein synthesis reactions are limited and carbon and energy are present in abundance. *Ralstonia eutropha* (also known as

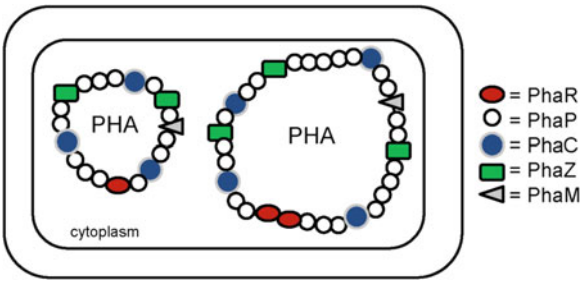


**Fig. 17.1** Schematic drawing of a *R. eutropha* cell containing PHB granules. Granules are surrounded by many different proteins, all with different functions in PHB homeostasis

*Cupriavidus necator*, formerly known as *Alcaligenes eutrophus*) has become the paradigm for study of PHA biosynthesis, largely because of its genetic tractability (Brigham et al. 2010; Budde et al. 2010; York et al. 2003) and that large amounts of intracellular PHA (≥80% of cell dry weight) are typically accumulated under nitrogen or phosphate deficiency (Anderson et al. 1990; Anderson and Dawes 1990). PHA is typically sequestered intracellularly in the form of inclusion bodies, termed granules. These granules are complex nutrient sequestration and mobilization organelles (Jendrossek 2009) that control the flow of carbon and reducing potential in the cell. The anatomy of a PHA granule in *R. eutropha* is shown in Fig. 17.1. Despite the large body of literature on the biology of the PHA granule, in *R. eutropha* there is still much to be learned about the roles of several granule-associated proteins (e.g. phasins PhaP2-4, and PHB depolymerases PhaZ2, PhaZ3, PhaZ5). PHA is a family of polymers with over 150 types known to exist (Valentin and Steinbüchel 1994), all differentiated by the monomers incorporated into them. Wild-type *R. eutropha* produces PHA containing short chain length (SCL) monomers like PHB (containing only the 3-hydroxybutyrate, or 3HB, monomer, Fig. 17.2) and P(HB-*co*-HV) (containing 3HB and 3-hydroxyvalerate, or 3HV, monomer, Fig. 17.2). Due to the ease of genetic manipulation of *R. eutropha* and a maturing set of genetic tools, strains can be constructed that will produce medium chain length (MCL) PHA and polymer containing monomers of mixed chain length (SCL and MCL) (Budde et al. 2011; Loo et al. 2005; Mifune et al. 2008; Riedel et al. 2012a).

*R. eutropha* is an industrially relevant organism, and some of its advantageous traits are discussed in Table 17.1. Due to its autotrophic growth capabilities, *R. eutropha* was first considered for $CO_2$ mitigation in space vessels for astronauts (Schlegel and Lafferty 1971). However, it is generally used for high productivity biosynthesis of PHA biopolymer. PHA has become attractive to the "green" materials industry due to the fact that it is bio-based, biodegradable, biocompatible, and its material properties are adjustable in similar profile to those of several petroleum-based plastics. Thus, PHA is considered a more environmentally friendly alternative to chemically synthesized plastics, and many applications (industrial, household, medical, etc.) are being sought for this class of polymer.

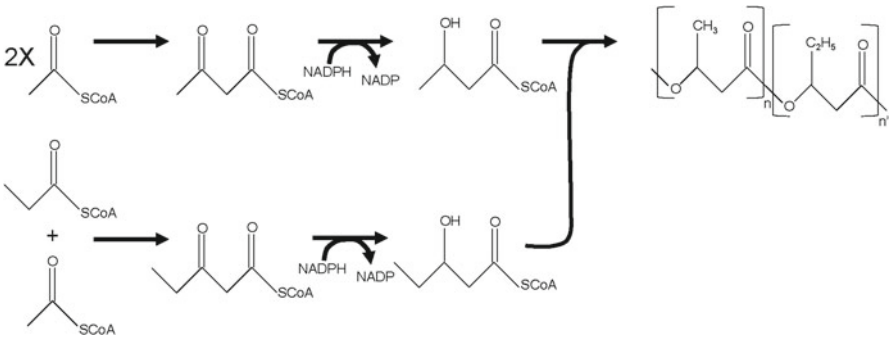


**Fig. 17.2** Wild-type *R. eutropha* is capable of producing short chain length (SCL) polyhydroxyalkanoates, like PHB or P(HB-*co*-HV). For PHB production (top reactions), two molecules of acetyl-CoA are ligated together to produce acetoacetyl-CoA, which is then reduced to form 3HB-CoA, the precursor for 3HB monomers. For 3HV monomer production (bottom reactions), acetyl-CoA and propionyl-CoA are ligated together and then reduced to produce 3HV-CoA

**Table 17.1** Characteristics of *Ralstonia eutropha* strains that make them suitable for use in industrial production of bio-based products

| Characteristic/trait | Relevance in biomaterials and bioproducts production | References |
|---|---|---|
| Genetically manipulable | Can construct *R. eutropha* strains that produce different types of bio-based compounds, including many types of PHA | Budde et al. (2011), Loo et al. (2005), Aboulmagd et al. (2001) |
| Carbon source utilization range | Can produce value added products using plant oils or other inexpensive carbon sources, like agricultural and food processing waste streams | Brigham et al. (2010), Bruland et al. (2009), Volova et al. (2001), Yang et al. (2010) |
| Robust carbon storage pathway | Can produce intracellular biopolymers with a high productivity and purity | Kahar et al. (2004), Reinecke and Steinbüchel (2009), Riedel et al. (2012a) |
| Adjustable polymer material properties | Can produce variations of polymer makeup with ranges of medium and longer length monomers through fermentation process controls | Kahar et al. (2004), Reinecke and Steinbüchel (2009), Riedel et al. (2012a) |
| Autotrophic growth | Can utilize $CO_2$ for production of biopolymers and other products | Ishizaki et al. (2001), Volova et al. (2001) |
| Non-pathogenic/bio-compatible | Can be used to produced medical compounds and biopolymers for medical materials and devices | Shishatskaya and Volova (2004), Shishatskaya et al. (2002a) |
| Resistant to some toxic compounds | Is carbon monoxide resistant (see below) and can produce biopolymers from toxic mixtures, like syngas; can also potentially produce biopolymers from phenol | Savalieva (1979), Volova et al. (1988a) |

**Table 17.2** Some of the *Ralstonia eutropha* carbon flux pathways and their relevance in biotechnological production of materials and chemicals

| Pathway | Relevance in biomaterials and bioproducts synthesis | References |
|---|---|---|
| Tricarboxylic acid (TCA) cycle | PHA biosynthesis; utilization of most carbon sources | Yu and Si (2004) |
| Calvin-Benson-Bassham cycle | Production of biomaterials and chemicals from $CO_2$ | Bowien and Kusian (2002) |
| Entner-Doudoroff pathway | Utilization of sugars for growth and product formation | Lee et al. (2003) |
| Fatty acid β-oxidation | Production of biomaterials and chemicals from triacylglycerols and fatty acids | Brigham et al. (2010) |
| Glyoxylate cycle | Utilization of acetate/acetyl-CoA (incl. β-oxidation byproducts) | Wang et al. (2003), Yu and Si (2004) |
| Branched chain amino acid biosynthesis | Production of branched carbon chain products (e.g. isobutanol) | Li et al. (2012), Brigham et al. (2012), Lu et al. (2012) |

Some of the important *R. eutropha* carbon flux pathways are listed in Table 17.2. These pathways come into play during production of biotechnologically relevant materials and/or chemicals. Alteration of carbon flux in *R. eutropha* does not occur solely due to the presence of heterologous genes and enzymes or the absence of chromosomally encoded pathway genes. A simple change in the sole carbon source of the bacteria will change the types of intermediates through which carbon flows. For example, PHA production using fructose or $CO_2$ as the sole carbon sources will result in production fructose 6-phosphate and fructose 1,6-bisphosphate intermediates through the pentose phosphate pathway and the Calvin-Benson-Bassham cycle, respectively, while PHA production using triacylglycerols will result in acetyl-CoA intermediates. Thus, we focus not only on genetic manipulations of *R. eutropha* that can alter the types and quantities of carbon-based intermediates in the cells, but also useful compound production and polymer makeup through different carbon sources that take advantage of some of the pathways listed in Table 17.2.

## 17.2 PHA Biosynthesis by *R. eutropha*

PHA can be produced by *R. eutropha* from various substrates, among them individual compounds (sugars, alcohols, organic acids, triacylglycerols); animal and plant oils (corn oil, lard, tallow, palm oil, palm kernel oil); and waste products of the alcohol, sugar, and hydrolysis industries and olive and palm oil production; etc. (Cromwick et al. 1996; Loo et al. 2005; Tanaka et al. 1995; Yang et al. 2010). As mentioned above, *R. eutropha* can grow and produce PHA autotrophically, using $CO_2$, $H_2$, and $O_2$ as the main growth substrates. A genome-scale metabolic network model has been constructed for *R. eutropha* (strain H16) PHB biosynthesis (Park et al. 2011), confirming the importance of culture pH and C/N ratio for optimal polymer production.

In this chapter, we discuss the many methods used to produce value added products from *R. eutropha* cultures, in particular discussions on feedstocks used and products made from these feedstocks. Manipulation of carbon flow in the organism is a hallmark of *R. eutropha* physiology studies. Two main methods are used for this carbon flux manipulation; genetic alteration of the genes, enzymes, and pathways present in the bacterium, or by changing the type and timing of feedstocks used to synthesize the product. From the summaries below, the versatility and industrial importance of *R. eutropha* and its metabolism is evident.

## 17.3 PHA Biosynthesis by *R. eutropha* from Syngas

One of the main principles of industrial biotechnology, feedstock availability, implies both the stable presence of economic material and the feasibility of rapid substitution of one feedstock with another, with minimal impact on the process technology or impairment of quality of the resultant product. Hydrogen-oxidizing bacteria like *R. eutropha* are grown on gases (carbon dioxide, oxygen, and hydrogen) that constitute the carbon and energy sources for the cell. A recent metabolic network reconstruction involving autotrophic growth of *R. eutropha* revealed that the growth rate of *R. eutropha* is sensitive to the $CO_2/O_2$ ratio in an autotrophic gas mixture (Park et al. 2011). Typically, hydrogen is present in vast excess, due to its poor solubility in aqueous solutions (Morinaga et al. 1978).

In an autotrophic gas substrate mixture, hydrogen is the component gas that is most difficult to obtain, and it is essential to find ways to lower its cost in order to develop hydrogen biosynthesis for practical purposes. Toward this end, various coals and coal byproducts have gained attention as substrates for biotechnological production (Fakoussa and Hofrichter 1999). Large reserves of coals and their relatively low cost make them very promising materials for the future large-scale industrial production of microbial bioplastics. It has been shown recently that the bacterium *Pseudomonas oleovorans* and *Rhodobacter ruber* can synthesise PHA polymers in growth media containing coal liquefaction products, i.e. mixtures of humic acids (Fuchtenbusch and Steinbüchel 1999). Using syngas as a growth substrate for hydrogen-oxidizing bacteria like *R. eutropha* is challenging due to the presence of a potentially inhibitory compound, carbon monoxide (CO). Among hydrogen bacteria there are unique organisms that are resistant to CO, including *R. eutropha* strain Z1 (Savalieva 1979; Volova et al. 1987) and its faster-growing variant, *R. eutropha* strain B5786 (Stasishina and Volova 1996).

Studies of *R. eutropha* growth and physico-chemical activities were performed on gaseous substrates containing carbon monoxide in order to determine if these strains could produce large quantities of PHA using gaseous substrates like syngas. While *R. eutropha* strains were shown to be able to grow in the presence of CO (Volova et al. 1988a, b), increases in CO concentrations in the growth media resulted in a decrease in the specific growth rate of the cells, increases in the activities of

hydrogenase enzymes and cytochrome concentrations, and enlargement of the cell membrane system (Volova et al. 1980, 1993).

It has been demonstrated that CO does not exert any significant effect on physiological-biochemical parameters of the nitrogen-limited culture of *R. eutropha* B5786 and to prove that CO-treated culture can produce high yields of polyhydroxyalkanoates (see above). The fact that CO produces no adverse effect on the PHA biosynthesis machinery in *R. eutropha* cell cultures offers opportunities for producing these polymers from gaseous products derived from coal via thermal treatment, such as coal gasification products. However, syngas used for this purpose must conform to certain requirements. First of all is that the $H_2$/CO ratio in the gaseous substrate must amount to at least 3:1. Second, the syngas must be free of carcinogenic tarry substances and any other biologically hazardous compounds. And finally, the process of brown coal gasification must be economically acceptable.

Syngas can be produced either involving the use of pure oxygen or through a multistage gasification process. In the latter case, heat is supplied into the gasifier with some heat carrier (gas, ash, ceramic balls, etc.) or through the reactor wall. However, processes of the latter kind are of rather low intensity. Moreover, in low-temperature processes of brown coal gasification, tar and tar-like substances make up a large percentage of the product, and it is technically difficult to utilize them for growth substrates. For the industrial gasification processes (Lurgi, Winkler, Koppers-Totzek processes) the CO:$H_2$ ratio is (48–58):(25–35). This is significantly different from the ratio required for PHA biosynthesis. Researchers proposed several methods for modifying the process of producing syngas and gas/vapor fuel mixture (Kuznetsov et al. 1995; Kuznetsov and Shchipko 1996). They attempted to optimize the process of gasification of the Kansk-Achinsk brown coal to produce syngas that could be used for synthesis of polyhydroxyalkanoates. This process consists of two main phases: oxidizing pyrolysis of coal and gasification of the resulting coal char by water vapor (Shchipko et al. 2003).

As the gas mixture for PHA biosynthesis must contain higher hydrogen concentrations, experiments were performed in which syngas with water vapor was converted into $CO_2$ and $H_2$, performed with excess water vapor (30%) at a temperature of 300–350°C. Another method examined was to feed excess vapor to the gasification step. Temperature and vapor feeding rates were regulated in such a way as to ensure that in the gas flux exiting the gasifier the ratio CO:$H_2O$ was equal to 1:3 to 1:5. This method yielded the syngas of the necessary composition without involving the additional stage of catalytic conversion of CO, but the heat efficiency of this gasification process was significantly lower, due to large heat loss with excess vapor. Carbon dioxide was extracted using potassium hydroxide and was then used to for bacterial growth. Studies in which the hydrogenous products of brown coal gasification were used as substrate for growing cells of *R.eutropha* B5786 proved that they could be used in biotechnological processes (e.g. PHA production). PHA specimens synthesized in the presence of CO had a high molecular mass, independent of CO concentration. The temperature parameters of the PHA and its crystallinity degree were also similar to those of the polymers synthesized from electrolytic hydrogen. This new technology of coal gasification does not require the use of

supplemental oxygen, which can be very costly. Thus, the resulting syngas is purer than any other syngas derived from brown coal by gasification techniques. These advantages are attained through uniting of two processes: oxidizing pyrolysis of pulverized coal in the fluidized bed of open-hearth or boiler slag and gasification of the resulting coal char using water vapor, performed in one technological cycle (Shchipko et al. 2003).

As established by the works discussed above, *R. eutropha* was able to grow and produce 70–75% of PHA when grown on a syngas substrate, with stoichiometry of gas consumption, polymer yield, and total productivity unaffected by the presence of CO (Volova and Voinov 2003, 2004). However, CO concentration did affect the rate of consumption of gas components by the culture. This value was determined from the following equation:

$$q = A \exp\left(0.024 \cdot X_{co}\right)$$

where q is specific rate of gas substrate consumption, kg/(kg h); $X_{CO}$ is volumetric concentration of carbon monoxide in the gas mixture, %; A is coefficient equal to 0.036 for hydrogen, 0.11 for carbon dioxide, and 0.17 for oxygen. Rates of gas consumption increased with increasing CO concentrations in the growth medium. The rate of gaseous substrate consumption by *R. eutropha* was only affected by CO during growth, and not during PHA production (Volova and Voinov 2003).

Given the findings discussed in the aforementioned works, CO should be rather regarded as an extraneous inhibitor. As has been previously shown (Volova et al. 1985), its oxidation rate by this culture is low (30–50 μM CO/min·g protein). Thus, as oxygen, carbon monoxide, and particularly hydrogen were consumed, CO concentration of the culture increased, while the partial pressure of the other gases decreased. This decreased the solubility of gas components in the liquid. As a result, in the media containing large amounts of CO, in the first stage, as the density of bacterial suspension increased, the hydrogen concentration in the culture decreased and even reached critical values. Thus, CO should be flushed from the culture and replaced with fresh syngas substrate. In order to minimize CO concentrations and optimize growth substrate gas concentrations in the culture, different modes of gas supply to the culture have been tested in experiments: (1) a single injection of gas into the culture; (2) recirculation of the gas mixture and ejection of part of the gas; and (3) complete utilization of the gas substrate and degassing of the culture medium to remove unutilized gases (Volova and Voinov 2003). The third option involving complete utilization of gas and degassing to remove extraneous CO yielded the best results. Under these gas exchange conditions, the 70-h fermentation cycle yielded 22 g biomass/L, with PHA concentration 75%, with hydrogen and oxygen utilization reaching 90%. Thus, with the same final PHA concentration and biomass production attained for the same culture period, variations in gas supply conditions significantly increase the completeness of gas substrate utilization, which minimizes costs.

These findings made it possible, for the first time in practical biotechnology, to synthesize high yields of polyhydroxyalkanoates from products derived from brown coals via modified gasification procedure. PHA biotechnology on syngas has been protected by RF Patent No. 2207375.

## 17.4 Synthesis of PHA Copolymers and Their Characterization

PHB was the first characterized of all the polyhydroxyalkanoates, but, in general, it does not have good thermoplastic properties. PHB is a very crystalline material that although strong, is also brittle. Mechanical and thermal properties of PHA can be improved as 3HB monomers are co-polymerized with longer chain length monomers, like 3HV or 3HHx. These copolymers typically exhibit better tensile strength, improved elongation to break, larger thermal processing window, and many other characteristics that are more favorable for a wide variety of applications (Noda et al. 2005).

Wild-type *R. eutropha* is capable of producing P(HB-*co*-HV) in the presence of 3HV precursor molecules, like propionate (Du et al. 2001) or valerate (Shang et al. 2004). Both propionate and valerate are toxic to *R. eutropha* at high concentrations (>0.4%, w/v), resulting in inhibition of growth and PHA production. For optimal P(HB-*co*-HV) biosynthesis by *R. eutropha*, feeding strategies must be developed for P(HB-*co*-HV) production (Du et al. 2001; Shang et al. 2004; Yu et al. 2005). Growth and PHA production on a mixture of organic acids derived from agricultural waste streams have been examined, resulting in production of a value added product from waste streams using wild type *R. eutropha* (Hassan et al. 2002; Yee et al. 2003). Following these works, a mixture analysis was developed for adjusting the ratios of the organic acids acetate, propionate, and butyrate to produce polymers that are "tailor made" with different 3HB and 3HV monomer contents (Yang et al. 2010), thus resulting in polymers with different properties (Fig. 17.3). P(HB-*co*-HV) containing a high molar % 3HV monomer has also been achieved with the aid of strain engineering. An *R. eutropha* strain expressing *phaC* and *zwf* (encoding a glucose 6P dehydrogenase) was shown to produce P(HB-*co*-58 mol%HV) when fed only 0.5% valerate as a 3HV precursor (Choi et al. 2003).

P(HB-co-HV) has been produced in fed-batch cultures of *R. eutropha* with productivities of up to 1.6 g PHA/L/h by employing various feeding strategies. Thermal and mechanical properties of resulting copolymers were tested, and it was determined that glass transition temperatures of P(HB-*co*-HV) copolymers were lower than PHB homopolymer. The effects of polymer aging on its mechanical properties were also tested and elongation to break values were shown to decrease as the polymer aged from 0 to 28 days (Madden and Anderson 1998). This is presumably because of crystallization occurring in the polymer over time. Aging effects resulting in brittleness of copolymer were witnessed in early works using commercial samples of P(HB-*co*-30%HV) (Scandola et al. 1989). Similar results have been seen in PHB (de Koning et al. 1992; Scandola et al. 1989), and also presumably from crystal formation in the polymer.

Even though copolymerization of 3HB and 3HV monomers offers improved properties of the resulting polymer, the incorporation of MCL monomers along with 3HB in PHA results in more dramatic improvement of the polymer properties (Budde et al. 2011; Noda et al. 2005). In the class of copolyesters of SCL and MCL PHA, the best studied member is poly(hydroxybutyrate-*co*-hydroxyhexanoate) (P(HB-co-HHx)). This P(HB-co-HHx) has a lower melting temperature, broader

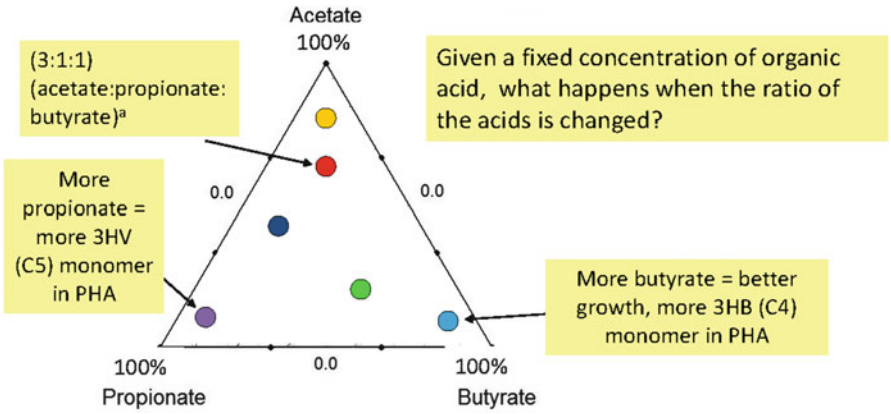


**Fig. 17.3** Schematic of organic acid feedstock mixture analysis for P(HB-*co*-HV) production by *R. eutropha* cultures. Altering components in the ratio of an acetate, propionate, and butyrate mixture results in altered growth kinetics or PHA composition. Software used for initial analyses (Yang et al. 2010) can be found at the website http://www.statsoft.nl and http://www.minitab.com. [a]The original acetate, propionate, and butyrate ratio was published in the reference (Yee et al. 2003)

thermal processing window, lower Young's modulus, and a longer elongation to break than either PHB or P(HB-*co*-HV) (Noda et al. 2005), indicating that it is tougher and more flexible than either PHB or P(HB-*co*-HV). As with P(HB-*co*-HV), the thermal and mechanical properties of P(HB-*co*-HHx) depend on the amount of other (non-3HB) monomers present in the polymer (Brigham et al. 2011).

Several groups have produced P(HB-*co*-HHx) from engineered *R. eutropha* strains. Many of these engineered *R. eutropha* strains use the broad substrate specificity PHA synthase gene (*phaC*) from *Aeromonas caviae* (Kahar et al. 2004; Loo et al. 2005; Mifune et al. 2008). These papers discussed no other genetic modifications to *R. eutropha* beyond addition of the *A. caviae phaC* gene and/or inactivation of *R. eutropha*'s native *phaC* gene. With this modification, the maximum amount of 3HHx monomer present in the resulting copolymer was determined to be 5 mol% (Kahar et al. 2004; Loo et al. 2005). Other synthase genes that have been used for production of P(HB-co-HHx) by *R. eutropha* include *phaC* from *Pseudomonas fluorescens*, which also resulted in the biosynthesis of P(HB-*co*-5 mol%HHx) (Noda et al. 2005). P(HB-co-HHx) was also produced by engineered *R. eutropha* grown on fructose. In this engineered strain, PHA synthase and an *(R)*-specific enoyl-coA hydratase gene (*phaJ*) from *A. caviae* were used, along with a crotonyl-CoA reductase gene from *Streptomyces cinnamonensis* (Fig. 17.4a). In this work, the 3HHx-CoA precursor was synthesized *de novo* by the engineered *R. eutropha* strain and incorporated into PHA. The resulting HHx content in polymer was demonstrated to be ~2 mol% (Fukui et al. 2002). An earlier work had demonstrated de novo biosynthesis of MCL PHA precursors in *R. eutropha* strains expressing a PHA synthase gene and a 3-hydroxyacyl-ACP transferase gene (*phaG*) from *Pseudomonas* sp. (Matsumoto et al. 2001). Recently, *R. eutropha* strains were engineered to

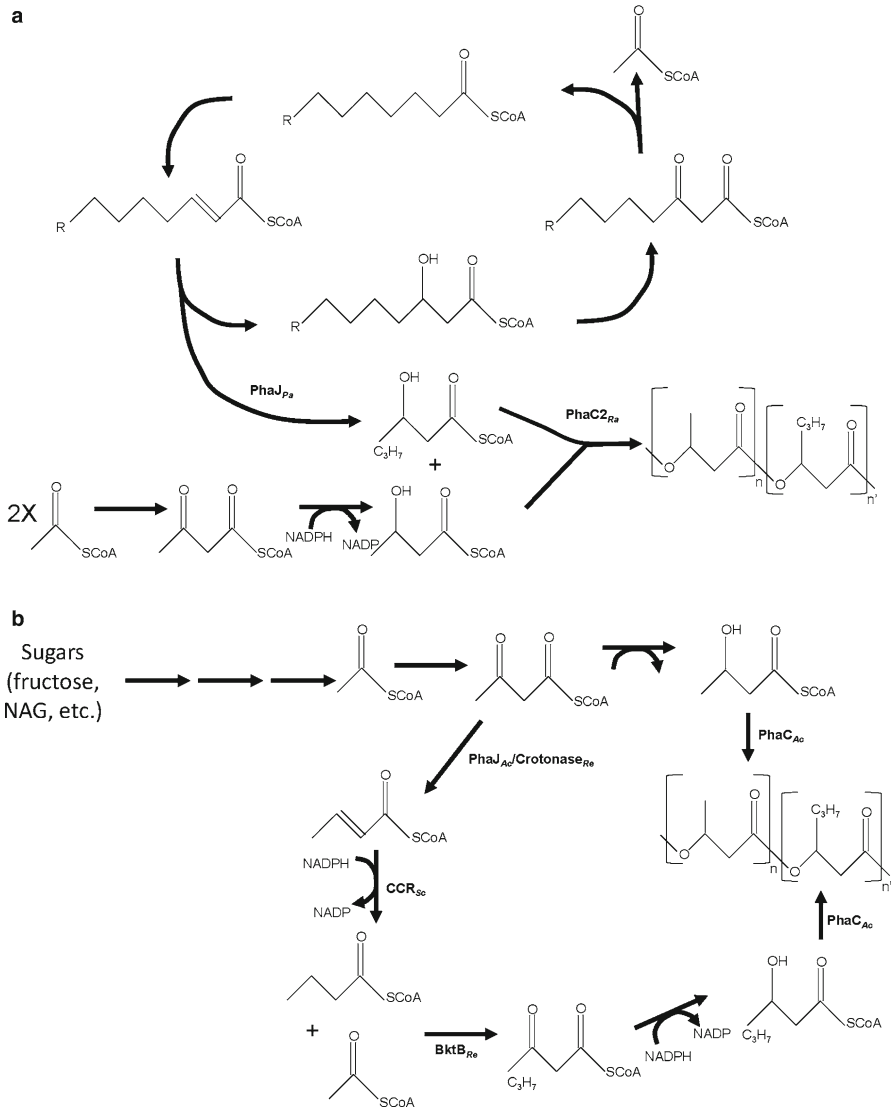


**Fig. 17.4** (**a**) Schematic of P(HB-*co*-HHx) biosynthesis of *R. eutropha* strains capable of producing *de novo* HHx monomers. (**b**) Schematic of P(HB-*co*-HHx) biosynthesis of *R. eutropha* strains that produce high levels of HHx monomers from fatty acid beta-oxidation intermediates. *R. eutropha* strain in (**a**) was engineered by Budde et al. (2011), and *R. eutropha* strain in (**b**) was engineered by Fukui et al. (2002)

produce copolymer with >15 mol% HHx incorporated, when these strains were grown on plant oils (Budde et al. 2011). The schematic of the engineered P(HB-*co*-HHx) pathway is shown in Fig. 17.4b. In fed batch fermentations with palm oil as the sole carbon source using these strains, >140 g/L dried biomass was produced

containing >70% PHA. This polymer contained 17mol% 3HHx monomer. Productivity of fed batch fermentations was greater than 1 g PHA/L/h (Riedel et al. 2012), suggesting that this P(HB-*co*-HHx) production using engineered *R. eutropha* as the biocatalyst is a scalable process.

The copolymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB)) has also been produced by *R. eutropha* and has potential medical applications. As with other copolymers, thermal properties of P(3HB-co-4HB) improve with increasing amounts of 4HB monomer (Ishida et al. 2001; Kasuya et al. 1996). There are many instances in the literature where P(3HB-*co*-4HB) is produced (Cavalheiro et al. 2012; Ishida et al. 2001; Kim et al. 2005; Volova et al. 2011), and in most cases, a 4HB precursor molecule (γ-butyrolactone, 4-hydroxybutyrate, etc.) is typically added to the culture. Depending on the amount of precursor feeding, the 4HB fraction of PHA produced by *R. eutropha* can vary from 0 to 100 mol% (Cavalheiro et al. 2012; Ishida et al. 2001; Kim et al. 2005; Volova et al. 2011).

Other types of PHA polymers have been produced by *R. eutropha* in the laboratory. The 3-hydroxy-4-methyl valerate (3H4MV) monomer was incorporated into PHA from *R. eutropha* expressing a modified *phaC* gene from *Pseudomonas* sp. 61–3 (Tanadchangsaeng et al. 2009). Further studies indicated that leucine can be fed to cells as a precursor for 3H4MV, and the mol% of 3H4MV could be enhanced in PHA biosynthesis when leucine analog resistant mutant strains were used (Saika et al. 2011). The high 3H4MV polymers exhibited lower melting temperatures than either PHB or P(HB-*co*-HV), but along with this, lower molecular weight.

## 17.5 Pilot Scale Production of PHA

The palm oil industry is one of the biggest industries in Malaysia. With millions of tons of natural products (palm oil and palm oil products) and even larger waste streams (palm oil mill effluent, or POME) available (Hassan et al. 2002), there is strong incentive to produce scalable processes for PHA production using these carbon-rich streams as feedstocks. The Malaysia and MIT Biotechnology Partnership Programme (MMBPP) has undertaken this task with great success. The culmination of the group's studies of *R. eutropha* production strain construction (Budde et al. 2011), fermentation process control (Riedel et al. 2012a), and polymer recovery (Riedel et al. 2012b) was the design and construction of a pilot scale biopolymer production facility in 2011 by SIRIM Berhad, outside of Kuala Lumpur, Malaysia. The pilot plant has a 2,000 L capacity and the ability to produce various PHA polymers from palm oil products and treated POME. With bioplastics production using robust strains and plentiful carbon feedstocks, it is believed that the cost of production can be lowered significantly, down to almost USD$2 per kg (Ismail 2011).

Testing of these PHA production technologies in the Pilot Production Facility is a way to determine more accurately physicochemical parameters of fermentation, calculate the material and energy balance, work out technological regulations, and prepare sufficient amounts of the product for investigations. In a similar effort

to study scaleup of PHA production processes on various feedstocks, the first Russian pilot facility for researching PHA production was constructed and launched in January 2005 at the Krasnoyarsk Research Center of the Siberian Branch of the Russian Academy of Sciences. That facility was the outcome of cooperation between researchers of the Institute of Biophysics SB RAS and specialists of the Biotechnology Department at Biokhimmash company of Moscow, within the framework of the International Science & Technology Center project (Volova et al. 2006b).

The facility is based on the previously developed technology and can produce sufficient quantities of polymers to satisfy the requirements of all Russian research institutes and to perfect the technologies of manufacturing special polymer items. Furthermore, it can serve as a tool to optimize polyhydroxyalkanoate production technology: using new feedstocks, reducing the cost of the polymer, broadening the range of the synthesized polyhydroxyalkanoates, and obtaining the data for the further scale-up of the process to the commercial level. The pilot lots of variously structured polymers were used to develop the techniques and procedures of processing the polymers into various items. PHAs in different phase states proved to be usable for the production of films, 3D membranes, sutures, microparticles, and composites. PHAs and PHA-based items were examined in biomedical studies, exhibiting good functional properties, and proved to be biomedical grade materials (Volova et al. 2006a).

## 17.6 Applications of PHA Polymers

As mentioned above, PHA polymers of all types offer a biocompatible and biodegradable alternative to petroleum-based plastics in many applications. Household applications include linings of milk cartons and diapers, while agricultural applications include biodegradable mulch films and coatings for fertilizer pellets (Philip et al. 2007). Metabolix, Inc., from Cambridge, MA, USA produces a wide range of biodegradable PHA-based products under the name Mirel™. Such products include packaging materials, gift cards, erosion control devices, and compost bags (www.metabolix.com). Medical applications of PHA polymers are currently being tested, as discussed in detail below. PHAs biocompatibility means that it can be used in contact with animal tissues, blood, etc. Tepha, Inc., of Cambridge, MA, USA manufactures several medical materials and devices from PHA, such as surgical meshes and films. These medical products have been demonstrated to have favorable mechanical properties for use in surgical procedures and have become widely adopted, particularly in Europe (www.tepha.com).

Recent advances in transplant surgery and introduction of new materials in medicine have made biological safety of medical items, such as primarily implants, a critical issue. The materials for fabricating temporary implants (sutures, artificial pericardia, stents, etc.) must be not only biocompatible, but also prone to biodegradation, forming products that are non-toxic to the organism. Finding materials possessing these balanced properties is a great challenge. Implants made from biodegradable materials

are designed to repair tissues or organs, promote the growth of the surrounding tissues, and to be controllably degraded once they have been replaced by native biological structures.

It is generally recognized that biomedical-grade materials, intended to contact living organisms, must possess a complex set of necessary biological and physical-mechanical properties. They must be biocompatible at the level of cultured cells and tissues of the macroorganism and non-toxic both before and after degradation. These materials must also fulfill a supportive function for cells, favor their proliferation and differentiation, allow for availability of growth substrates, and allow for release of metabolites. The material must possess proper mechanical strength and flexibility, have tissue growth favorable surface characteristics, be tolerant of conventional sterilization methods and the effects of aggressive biological media, while also being easy to process using conventional production methods. Comprehensive preclinical investigations of polyhydroxyalkanoates, including in vitro assay systems and short- and long-term exposure experiments on laboratory animals, have shown that PHAs of different compositions, in this case produced by and purified from *R. eutropha* strains, can be regarded as medical grade materials. Polymers and polymeric items, such as film-based tissue engineering scaffolds and suture fibers, were evaluated in conventional and sanitary chemical, toxicological, and biomedical tests. The results of the tests demonstrated that the two main PHA types studied, PHB and P(HB-*co*-HV) both of *R. eutropha* origin, are highly biocompatible at the level of the cell, tissue, and macroorganism, and can be used in contact with blood (Sevastianov et al. 2003; Shishatskaya et al. 2002b, 2004, 2008; Volova et al. 2003).

To choose proper sterilization techniques, PHAs were tested for their heat resistance and radiation tolerance. PHA films, membranes, and sutures subjected to sterilization by conventional techniques did not exhibit any changes in their structure or function, suggesting a conclusion that PHAs can be sterilized using various conventional techniques: dry heat, autoclaving, disinfectant solutions, and γ-irradiation.

A very important issue is mechanism and kinetics of degradation of resorbable materials in biological media. Investigations of PHA degradation were conducted in biological media in vitro (in stabilized blood and serum) and in vivo with implants (films, sutures, and microparticles) immediately contacting with animal tissues and with implants enclosed in diffusion chambers. This in vivo process did not allow fibrous capsules to be formed and provided conditions for investigating biocompatibility and biostability of the material in terms of true cellular reaction. The PHAs used in this study were found to be degraded in biological media at low rates (over months and years) via humoral and cellular pathways, involving macrophages and foreign body giant cells with a high activity of acid phosphatase. It was also found that PHA biodegradation rate depends on the chemical structure of the polymer, the form of the item and implantation site (Shishatskaya et al. 2002a, b, 2005).

As previously mentioned, an important property of PHAs is their manufacturing processability to form special items from different phase states, using conventional techniques, without adding processing aids or plasticizers (Amass et al. 1998). Having investigated the dissolution and melting behavior of PHAs and physicochemical

properties of polymer solutions, gels, and melts, a series of various 2D and 3D matrices were prepared for cell cultures: flexible films and porous membranes, monofilament fibers between 0.15 and 0.40 mm in diameter, ultrathin fibers between 1 and 5 μm in diameter, microparticles, sponges, solid and porous 3D matrices, as well as composites with hydroxyapatite and collagen.

PHA solutions of various densities were used to prepare transparent flexible films. The surface properties of PHB and P(HB-*co*-HV) film scaffolds were similar to each other and to those of synthetic polyesters (polyethylene terephthalate, poly (methyl methacrylate), polyvinyl chloride, and polyethylene) (Shishatskaya 2007). The scaffold's surface properties are important for cell attachment and proliferation. To enhance cell adhesion to the surface, improve the gas-dynamic properties of scaffolds, and increase their permeability for substrates and cell metabolites, the scaffolds can be treated by physical factors or by chemical reagents. Biocompatibility of PHA scaffolds has been enhanced by immobilizing collagen film matrices on the scaffold surface and coating with chitosan and chitosan/polysaccharides (Hu et al. 2003).

One of the approaches to modification of PHA surfaces is to treat them with gas plasma. A more recent way to modify PHA properties is to use laser-cutting technique. The advantage of this technique is that the surface can be modified without destroying the material and releasing toxic byproducts. Laser processing has been shown to enhance the adhesive properties of the PHA matrix surface. PHA film scaffolds were laser processed and, since membranes are transparent in the near and far IR spectral regions, they can be processed using lasers that generate radiation of a far IR wavelength. Using a $CO_2$-laser with power ranging from 3.0 to 30.0 W, a series of films was prepared, with surface properties modified from pronounced roughness to perforations. The microstructure and surface properties of the film scaffolds were examined and it was observed that water contact angles were reduced to 50% and scaffold hydrophilic properties were improved without destroying the structure of the polymer. The adhesion of fibroblasts and osteoblasts to such scaffolds was 18% higher than to unprocessed ones (Shishatskaya 2007).

Cell culture scaffolds require mechanical strength and high bulk porosity. PHAs have the former, but one way to increase the porosity of a matrix is by fabrication using two-component mixtures. One method for fabrication of porous polymeric scaffolds is via a solution of two-component mixtures, containing a water-soluble component and a water-insoluble compound, and then to leach out one of the components from the scaffold in the solution. Leaching techniques usually involve the use of water-soluble sodium chloride or sucrose crystals. By varying the soluble compound concentrations, one can control the total porosity and the pore sizes in the scaffolds. Porous scaffolds can also be prepared using three-component mixtures containing polymer solvents and nonsolvents. These three-component systems can be used to prepare high-porosity polymer scaffolds with pore sizes <5–10 μm (Yang et al. 2001).

PHA membranes of different compositions have been constructed and characterized, including PHB, P(3HB-*co*-4HB), P(HB-*co*-HV), and P(HB-*co*-HHx), all of *R. eutropha* origin. Examination of microstructure of the surface of the membranes

revealed that membranes prepared from P(HB-*co*-HHx) had the roughest surface, and those prepared from P(HB-*co*-HV) had the smoothest surface. Copolymer membranes had smaller water contact angles and higher hydrophilicity than membranes prepared from highly-crystalline PHB. Testing biocompatibility of these scaffolds, mouse fibroblast NIH 3T3 cells were cultivated on PHA membranes, and results Romanovsky-type staining, DAPI staining, and the MTT assay showed that membranes prepared from PHAs of different chemical compositions did not exhibit cytotoxicity to cells cultured on them and proved to be highly biocompatible. Cell attachment and proliferation on PHA membranes were similar to those on polystyrene and better than those on membranes prepared from polylactic acid (PLA). These observations suggest that PHAs are biocompatible can be used as matrices on which to grow cell cultures. Among promising approaches to preparing ultrafine fibers, membranes, and micro- and nano-particles from PHA as models of cell scaffolds are nanotechnological methods such as microencapsulation and electrostatic spinning. Electrostatic spinning technique has been used to prepare ultrafine PHA fibers. These fibers, with diameters from 1 to 5 μm were used as scaffolds for growing mouse fibroblasts (NIH 3 T3). The results showed that a significant number of cells adhered to the fibers and proliferative activity was robust. Microencapsulation techniques, which have been developed in recent years (Sayin et al. 2006), are used to prepare micro- and nano-size PHA particles with large surface area for use as controlled drug delivery systems and cell culture scaffolds. Biocompatibility of the microparticles was demonstrated in cell cultures and in experiments with animals that received them via injection (Shishatskaya et al. 2009).

Development of cellular and tissue engineering offers new opportunities to reconstructive orthopedics. One of the approaches to improving mechanical properties of hydroxyapatite (reducing rigidity, increasing elasticity) is preparation of hydroxyapatite/synthetic polymer hybrids. A novel approach involves fabrication of hybrid materials based on hydroxyapatite and biodegradable polymers. As in vivo bioresorption rates of PHA are several times lower than those of other known biodegradable biomaterials (polylactide, polyglycolide), these polyesters can be used for prolonged regeneration of large bone defects. Hybrid matrices of PHB and hydroxyapatite were seeded with osteoblastic cells. Biocompatibility and functional properties of these matrices were confirmed both in vitro and in vivo, suggesting that PHB/HA hybrid 3D matrices have good osteogenic potential, facilitate bone formation, and can be used in further studies as bioactive constructs for bone defect repair (Shishatskaya 2006).

PHAs have also been studied as matrices for holding and delivering drugs or in controlled drug delivery systems. The most promising drug delivery systems seem to be polymer microparticles of diameters from 0.5 to 5.0 μm, which can function in vivo for up to 12 weeks and can be injected intramuscularly, intraperitoneally, and intravenously (Shishatskaya 2007; Shishatskaya et al. 2008). An experimental prolonged-action system in the form of microparticles demonstrated that a stable concentration of drug, delivered from a PHA matrix, in the blood and peritoneal fluid could be maintained for 10 days. It was also found that the experimental form of

rubomycin hydrochloride encapsulated into PHA microspheres and intraperitoneally injected to laboratory mice that had also been inoculated with a 100% lethal dose of Ehrlich ascites carcinoma cells significantly inhibited proliferation activity of carcinoma cells and enhanced the survival of tumor-bearing mice to 40% (Shishatskaya et al. 2008).

The use of PHA coatings to enhance biocompatibility of vascular stents was shown to be an effective modification. Self-expanding nitinol mesh vascular stents were coated with PHA and PHA loaded with an antiproliferative drug. Stents were placed in the femoral artery of dogs and examined for 120 days. The analysis of the state of the vessels and morphometric examination showed the effectiveness of coating vascular stents with PHA, especially PHA loaded with an antiproliferative drug. Reactive changes in the vessel wall were less pronounced and no complications occurred that are usually caused by implantation of uncoated metal stents (Protopopov et al. 2005, 2008).

Also, fully resorbable PHA (mesh, spiral, and tubular) stents were constructed and tested as implants for endobiliary surgery in laboratory animals (Markelova et al. 2008). The stents were implanted to the supraduodenal part of the bile duct of laboratory animals, fixed with PHA sutures, and monitored for 120 days. In the study, bilirubin levels of animals remained unimpaired, no unfavorable tissue responses to the implant were registered, the bile duct lumens at the implantation sites remained unchanged and retained their pre-surgery sizes, and no deformities or inflammations were observed at the site of implantation. Histological examination of the liver and the biliary tract did not reveal any unfavorable effects of stenting. These results suggest biocompatibility for the designed stents and provided a basis for local clinical trials.

PHA polymers recovered from cultures of *R. eutropha* have tremendous potential for development of medical technologies, including surgical reconstruction, orthopedic and trauma surgery, cardiovascular and abdominal surgery, and pharmacology. The matrices discussed in this section are biocompatible and show promise in many types of medical applications. PHA can be the next generation of medical material, with studies of polymer recovered *R. eutropha* at the forefront of the promising, burgeoning medical PHA field.

## 17.7 Manipulation of Carbon Flux in *R. eutropha* for Biosynthesis of Other Useful Compounds

As discussed in previous sections, *R. eutropha* is most widely studied for its ability to produce polyhydroxyalkanoates. However, given the ease of genetic manipulation and ability to utilize a wide range of carbon substrates, *R. eutropha* is an industrially relevant organism that can be designed to produce several important and value added natural products. Cyanophycin, a biodegradable alternative to polyacrylate (Aboulmagd et al. 2001; Schwamborn 1998), is typically synthesized by cyanobacteria albeit with low yields. Engineered *R. eutropha* strains have been designed to produce

intracellular cyanophycin, and can accumulate up to 32% of their cell dry weight with the compound (Voss and Steinbüchel 2006). Furthermore, high cell density cultures have been achieved using a modified cyanophycin gene expression system in recombinant *R. eutropha* (Lin et al. 2012). Self-assembling peptides can form nanofilament-like structures and have also been considered for tissue engineering. Reed, et al. have engineered *R. eutropha* strains to produce the RAD16-I::E peptide, which were purified from cells by fusion to cellulose binding modules (Reed et al. 2006).

With increased interest in microbial biofuels production, researchers are looking to *R. eutropha* as a recombinant fuels and chemicals producing organism. Utilizing the organism's branched chain amino acid biosynthetic pathway, a heterologously expressed decarboxylase gene (de la Plaza et al. 2004), and native alcohol dehydrogenase enzymes (Jendrossek et al. 1990), *de novo* production of isobutanol has been achieved, with the long term goal of producing isobutanol biofuel autotrophically (Brigham et al. 2012; Li et al. 2012; Lu et al. 2012). Longer chain fuel molecules are being produced by groups who have altered the flow of carbon in *R. eutropha* through to fatty acid biosynthesis to produce fatty acids and fatty acid methyl esters for use as biofuels (www.opxbio.com; arpa-e.energy.gov/ProgramsProjects/Electrofuels.aspx).

A microbial-based process for vanillin production has been sought in the flavoring and food industries. Vanillin has been biosynthesized by recombinant *Escherichia coli* (Yamada et al. 2008), *Rhodococcus* (Plaggenborg et al. 2006), and *R. eutropha* (Overhage et al. 2002). In the latter work, ferulic acid, a precursor to vanillin biotransformation, was converted by engineered *R. eutropha* strains from eugenol as the precursor. The engineered strain expressed eugenol catabolic genes from *Pseudomonas* sp. HR199, and achieved a productivity of ferulic acid conversion of 2.9 mmol/L/h (98 mol% yield). The 3-hydroxybutyrate monomer (3HB) has been shown to exhibit antimicrobial, antiviral, and insecticidal activities, suggesting that 3HB has wide industrial and medical applications (Tokiwa and Ugwu 2007). *R. eutropha* was subjected to UV mutagenesis to select for mutant strains that secrete 3HB. Strains were discovered that secrete 3HB, and the mutants were reported to contain a disruption of *phaB* (Ugwu et al. 2008). However, since at least two different *phaB* mutations were shown to knock down PHB production significantly in *R. eutropha* (Budde et al. 2010), it is unclear if the authors of this study mapped the actual mutations in their 3HB secreting strains.

Lastly, 2-methylcitric acid is a compound considered to be of importance for pharmaceutical applications, and is an intermediate of propionate metabolism in *R. eutropha* (Bramer and Steinbüchel 2001). Maximal production of 2-methylcitric acid was predicted to occur when the *prpB*, *prpD*, and *acnM* genes, whose products are all involved in the consumption of 2-methylcitrate, were deleted in *R. eutropha* (Park et al. 2011). In gluconate grown cultures of *R. eutropha* strains harboring a deletion of *acnM* and an additional *prpC* gene, a maximum production of 60 mM 2-methylcitric acid was achieved (Ewering et al. 2006), validating the genome-scale reconstruction predictions.

## 17.8 Outlook

Like many soil microorganisms, *R. eutropha* is able to utilize a versatile array of carbon sources for growth and biopolymer production. *R. eutropha* is genetically manipulable, increasing its value as an industrial microorganism for production of biopolymer and other engineered bioproducts. Understanding the flow of carbon in this organism is crucial to increasing its effectiveness in biotechnological endeavors, including high productivities of PHA synthesis. Studies have shown that *R. eutropha* is capable of using carbonic waste streams for production of biopolymer (Hassan et al. 2002; Yang et al. 2010; Yee et al. 2003). Scaling up fermentation efforts using waste streams and biosynthesis of polymer or other value added product is crucial for lowering costs associated with bio-based production. More effort must be made to understand the basic mechanisms of carbon flow in the organism. Study of the basic science of the metabolism of *R. eutropha* is a must to understanding how to unlock its full biotechnological potential. Microarray and proteomic studies have been performed using *R. eutropha* cultures (Brigham et al. 2010; Peplinski et al. 2010; Schwartz et al. 2009), but an in-depth look into the metabolomics and enzymology of the organism must not be far behind.

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