Engineering the heterotrophic carbon sources utilization range of *Ralstonia eutropha* H16 for applications in biotechnology

Elena Volodina¹, Matthias Raberg¹, and Alexander Steinbüchel¹,²

¹Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Münster, Germany and ²Environmental Science Department, King Abdullahaz University, Jeddah, Saudi Arabia

**Abstract**

*Ralstonia eutropha* H16 is an interesting candidate for the biotechnological production of polyesters consisting of hydroxy- and mercaptoalkanoates, and other compounds. It provides all the necessary characteristics, which are required for a biotechnological production strain. Due to its metabolic versatility, it can convert a broad range of renewable heterotrophic resources into diverse valuable compounds. High cell density fermentations of the non-pathogenic *R. eutropha* can be easily performed. Furthermore, this bacterium is accessible to engineering of its metabolism by genetic approaches having available a large repertoire of genetic tools. Since the complete genome sequence of *R. eutropha* H16 has become available, a variety of transcriptome, proteome and metabolome studies provided valuable data elucidating its complex metabolism and allowing a systematic biology approach. However, high production costs for bacterial large-scale production of biomass and biotechnologically valuable products are still an economic challenge. The application of inexpensive raw materials could significantly reduce the expenses. Therefore, the conversion of diverse substrates to polyhydroxyalkanoates by *R. eutropha* was steadily improved by optimization of cultivation conditions, mutagenesis and metabolic engineering. Industrial by-products and residual compounds like glycerol, and substrates containing high carbon content per weight like palm, soybean, corn oils as well as raw sugar-rich materials like molasses, starch and lignocellulose, are the most promising renewable substrates and were intensively studied.

**Keywords**

Carbohydrates, fatty acids, glycerol, metabolic engineering, PHAs, PTEs, *Ralstonia eutropha*, value-added bioproducts

**Introduction**

*Ralstonia eutropha* H16 is a Gram-negative, rod-shaped and facultative chemolithoautotrophic β-proteobacterium isolated from a spring in Germany. Initially described as *Hydrogenomonas eutropha* (Wilde, 1962), it has been the subject of frequent taxonomical reclassifications: *Alcaligenes eutrophus*, *R. eutropha*, *Wautersia eutropha*, and finally *Cupriavidus necator* (Reinecke & Steinbüchel, 2009). However, the designation *Ralstonia eutropha* remained the most established terminus in the scientific community and will be also used throughout this study.

As a ubiquitous inhabitant of soil and freshwater, *R. eutropha* is well adapted to constantly changing environments. Metabolic versatility of this microorganism made it to a model organism in microbiology concerning hydrogen-based chemolithoautotrophy and poly-3-hydroxybutyrate (P(3HB)) synthesis (Reinecke & Steinbüchel, 2009). Although aerobic respiration plays the major role in energy generation, *R. eutropha* can utilize nitrate and nitrite as alternative electron acceptors under anoxic conditions (Aragno & Schlegel, 1992, Pfitzner & Schlegel, 1973). During heterotrophic growth, diverse carbohydrates, lipids and organic acids serve as carbon and energy source (Figure 1), whereas in the absence of organic compounds a mixture of H₂, CO₂ and O₂ enables *R. eutropha* to grow autotrophically. In dependency on the situation in the habitat, *R. eutropha* can easily shift between autotrophy and heterotrophy (Kärst & Friedrich, 1984). Mixotrophical growth is observed when *R. eutropha* utilizes concomitantly organic and inorganic substrates as carbon and energy source (Friedrich et al., 1981). The Calvin–Benson–Bassham (CBB) cycle of *R. eutropha* is active in the polyhydroxyalkanoate (PHA) synthesis phase even during growth on fructose. On the other side, the complete tricarboxylic acid cycle (TCC) is also active during autotrophic growth (Schwartz et al., 2009; Shimizu et al., 2013).

During all nutrition modes, *R. eutropha* is able to store P(3HB). For this, two molecules of acetyl-CoA are condensed by a β-ketothiolase (PhaA) to acetooacetyl-CoA. The latter is reduced by the NADPH-dependent acetooacetyl-CoA reductase (PhaB) to (R)-3-hydroxybutyryl-CoA, which is further polymerized to P(3HB) by the PHA synthase (PhaC) (Anderson & Dawes, 1990). These three genes are organized in one operon *phaCAB*, which is constitutively expressed...
independent from the growth conditions (Peplinski et al., 2010). However, intensive synthesis of P(3HB) only starts if a carbon source is available in excess and if one essential nutritional element like N, O, S, Mg, K or P limits cell growth. P(3HB) can be stored up to 90% of the cell dry weight (CDW) by *R. eutropha* H16. Besides 3-hydroxybutyrate (3HB), other short- and medium-chain-length (SCL and MCL) hydroxyalkanoates as well as mercaptoalkanoates are polymerized by PhaC<sub>Re</sub> (Table S1; 24, 73).

Besides the academic interest, *R. eutropha* has found applications in industry for large-scale production of PHAs (Byrom, 1987). PHAs have attracted much interest due to their biodegradability and origin from renewable resources, as well as to thermoplastic/elastomeric properties similar to...
synthetic polymers (Steinbüchel & Valentin, 1995). With more than 150 different constituents, PHAs exhibit a broad spectrum of properties and functionalities (Chanprateep, 2010). Consequently, various technical and medical applications have been developed during the last decades (Anderson & Dawes, 1990; Hocking & Marchessault, 1994; Williams, 2005).

PHAs have been industrially produced since the 1970s (Imperial Chemical Industries (ICI); Monsanto:St. Louis, MO; Metabolix: Cambridge, MA; Zeneca: London, UK, etc.). However, the production costs of bioplastics are still 5–10 times higher than costs for the production of conventional plastics from petrochemicals (Rehm, 2010). The most critical aspect for industrial production bioproducts is their price competitiveness. Raw materials used for cultivation have an important impact on total production costs (Chanprateep, 2010). Inexpensive raw sugar-rich materials, such as molasses, starch and cellulosic resources, promise to reduce the production costs of microbially synthesized products. Industrial production of plant oils and biodiesel is accompanied with co-production of glycerol-, triacylglyceride- and free fatty acid (FA)-rich residuals as by-products (Figure S1). FA-rich feedstocks are substrates containing high carbon content per weight and can be transformed into biotechnologically relevant products with a very high yield (Sudesh et al., 2011). The versatility of R. eutropha's metabolism allows the utilization of a broad range of carbon sources. Further extension of the carbon substrate range as well as optimization of substrate conversion, are key for accelerating competitiveness of the gained products. Besides, the problem of organic wastes disposal can be partially solved by conversion of residuals to valuable products (Chanprateep, 2010).

Knowledge concerning PHAs and their microbial production is steadily expanding. Novel biopolymers of 3-mercaptoalkanoates and cyanopycin, low-molecular weight compounds and alcohols recently extended the range of products synthesized by R. eutropha (Grousseau et al., 2014; Li et al., 2012; Lütke-Eversloh et al., 2001; Shiraki et al., 2006; Voss & Steinbüchel, 2006). New information encouraged reviews every few years (Agnew & Pfleger, 2013; Braunegg et al., 1998; Chanprateep, 2010; Park et al., 2012; Steinbüchel & Valentín, 1995). Since the genome of R. eutropha H16 has been sequenced (Pohlmann et al., 2006), the energy metabolism of this bacterium has been reviewed (Cramm, 2009). The genetic potential of this bacterium as a model organism, the natural and engineered carbon storage compounds and alcohols recently extended the range of products synthesized by R. eutropha (Grousseau et al., 2014; Li et al., 2012; Lütke-Eversloh et al., 2001; Shiraki et al., 2006; Voss & Steinbüchel, 2006). New information encouraged reviews every few years (Agnew & Pfleger, 2013; Braunegg et al., 1998; Chanprateep, 2010; Park et al., 2012; Steinbüchel & Valentín, 1995). Since the genome of R. eutropha H16 has been sequenced (Pohlmann et al., 2006), the energy metabolism of this bacterium has been reviewed (Cramm, 2009). The genetic potential of this bacterium as a model organism, the natural and engineered carbon storage pathways for production of PHAs and other bioproducts by R. eutropha were analyzed (Brigham et al., 2012; Reinecke & Steinbüchel, 2009; Riedel et al., 2014; Steinbüchel & Lütke-Eversloh, 2003; Tsuge, 2002).

Recently, a variety of studies about the extension of the carbon source utilization range of R. eutropha and its optimization became available. To address these items, an understanding of the metabolic network of different renewable substrates concerning genomic and metabolic background of R. eutropha is essential. This stimulated to review the recent advances in this field with respect to the application of R. eutropha H16 wild type, mutants and metabolically engineered strains in biotechnology. The utilization of substrates that are mainly metabolized via the Entner-Doudoroff (ED)-pathway or are oxidized in β-oxidation cycle and TCC besides lignin derivatives, amino acids, sulfur compounds will be discussed and their impact on the synthesis of valuable products will be shortly reviewed.

**Utilization of gluconate and carbohydrates**

Besides gluconate only two carbohydrates, fructose and N-acetylglucosamine, are utilized as a carbon source by R. eutropha H16 (Figure 1; Kersters & De Ley, 1984). They are expensive, and their application is therefore economically not feasible for the production of cheap bulk products. Broadening the substrate range of R. eutropha will allow using inexpensive and renewable crop-derived feedstocks. Alternatively, second generation products, like lignocelluloses biomass and other agricultural wastes, provide a good alternative stock of carbon and energy for microbial conversions. Lignocellulose is the most abundant raw material composed of lignin (10–20%) and the carbohydrate polymers, cellulose (30–50%) and hemicelluloses (20–50%) (Figure S1; Keenan et al., 2006). Cellulose is a polysaccharide of glucose. Hemicellulose is a heteropolymer consisting of glucose, xylose, mannose, galactose, rhamnose, and arabinose. Celluloses and hemicelluloses can be microbially fermented or chemically hydrolyzed into monosaccharides, which can then serve as substrates for biotechnical production of value-added products (Limayem & Ricke, 2012).

**N-Acetylg glucosamine, fructose and gluconate**

Fructose, N-acetylg glucosamine and gluconate are catabolized in the ED-pathway with 2-keto-3-deoxy-6-phosphogluconate aldolase (EDA) as key enzyme, yielding pyruvate (Figure 1). Phosphofructokinase (Pfk) and 6-phosphogluconate dehydrogenase are absent in R. eutropha H16; therefore, the Embden–Meyerhof–Parnas (EMP)-pathway and the oxidative pentose phosphate pathways are incomplete. However, genes for an anaerobically operating EMP-pathway (glucoseogenesis) are present in R. eutropha (Pohlmann et al., 2006). The catabolic EMP-pathway was accomplished in R. eutropha by heterologous expression of pfk from Escherichia coli (Steinbüchel, 1986). Mutants with a defective ED-pathway serve as a suitable platform for the establishment of fructose utilization via the EMP-pathway, because the simultaneous function of EMP- and ED-paths in the wild type of R. eutropha had a negative effect on growth in presence of fructose. Alternatively, the ability of EDA-negative mutants to utilize fructose was restored via heterologous expression of the phosphoketolase gene from Bifidobacterium animalis (Feige et al., 2011). These experiments showed that central metabolic pathways can be switched off, while alternative routes can be easily established in R. eutropha. Pyruvate formed in the ED-pathway is further decarboxylated by the pyruvate dehydrogenase complex (PDHC) yielding acetyl coenzyme A (CoA). The latter is used in anabolic pathways for the synthesis of essential compounds, like amino acids and lipids, directed to P(3HB) synthesis and stored as a source of energy and carbon, or directly oxidized in TCC (Figure 1).

The amino sugar and glucose derivative N-acetylg glucosamine is transported by the phosphoenolpyruvate (PEP)-dependent phosphotransferase transport system (PTS\textsuperscript{NA})
into cells of *R. eutropha* (Figure 1). The responsible genes are organized in the *nag*FECABzwf1 cluster. N-Acetylglucosamine enters the cell through a porin (NagC) located in the outer membrane (Kaddor & Steinbüchel, 2011). Then, N-acetylglucosamine is phosphorylated by two proteins of the PTS\textsuperscript{Nag}, NagF and NagE. Therefore, PEP provides phosphoryl to the soluble NagF, which comprises three PTS domains, EF\textsuperscript{Nag}–HPr\textsuperscript{Nag}–EIIA\textsuperscript{Nag}. Phosphoryl is then transferred from NagF to a cytosolic domain EII\textsuperscript{Nag} and a membrane-spanning domain EIC\textsuperscript{Nag} of NagE. After phosphorylation of N-acetylglucosamine by NagE the acetyl-moiety is cleaved off by N-acetylglucosamine-6-phosphate deaminase (NagA), and glucosamine is further deaminated by glucosamine-6-phosphate deaminase (NagB) yielding fructose-6-phosphate. Fructose uptake is mediated by a CUT2 family ATP-binding cassette (ABC)-type transporter (frcACB) (Figure 1; Gottschalk, 1964; Pohlmann et al., 2006). Fructokinase catalyzes the phosphorylation of fructose. Fructose-6-phosphate, which is also obtained from N-acetylglucosamine, is then converted to glucose-6-phosphate by phosphoglucose isomerase and is further degraded in ED-pathway with gluconate-6-phosphate as one of the intermediates (Figure 1). If gluconate serves as the substrate, it enters the ED-pathway as gluconate-6-phosphate. The gluconate transport is mediated by a gluconate-H\textsuperscript{+} symporter catalyzed by a gluconate permease (GntP) and a gluconate transporter (GntT) (Kaddor & Steinbüchel, 2011).

Although *R. eutropha* H16 harbors only one specific transport system either for fructose or N-acetylglucosamine, an additional truncated PTS comprises a variety of PEP-PTS homologous proteins, like PtsN, PtsM, PtsH, and PtsI (Pohlmann et al., 2006). Recently, it was found that the truncated PTS is indirectly involved in the complex sugar transport system, regulatory functions of carbon and P(3HB) metabolism (Kaddor & Steinbüchel, 2011; Karstens et al., 2014). Diminished P(3HB) contents and facilitated P(3HB) transport were observed in PtsM, PtsH, and ptsI single, double, and triple mutants of *R. eutropha* (Kaddor & Steinbüchel, 2011). The lack of PtsI and PtsH impairs the transfer of a phosphoryl group to PtsN, as consequence the non-phosphorylated PtsN leads to a lower P(3HB) content. A deletion of ptsN had an opposite effect and led to an increased P(3HB) content in the cells if they were grown on gluconate. Further findings demonstrated that protein–protein interactions of non-phosphorylated PtsN with the key enzyme of a stringent response SpoT1 (GTP diphosphokinase/ppGpP hydrolase) can indirectly alter the global gene expression in the cell (Karstens et al., 2014). Thus, PtsN, in association with SpoT1, was found to participate in the regulation of a stringent response in *R. eutropha* and consequently influences the P(3HB) content.

**Glucose**

The wild type of *R. eutropha* is unable to utilize glucose as a carbon source. However, glucose-6-phosphate is a common intermediate of the ED-pathway (Figure 1). Crude extracts of fructose-grown cells of strain H16 exhibit hexokinase activity, which phosphorylates besides fructose, also mannose and glucose (Gottschalk, 1964). Furthermore, it was demonstrated, that \(^{14}\)C glucose does not enter the cells. *R. eutropha* H16’s behavior was designated as cryptic with regard to glucose, i.e. even cells, which were grown with fructose and equipped with all enzymes necessary for glucose degradation, were unable to metabolize glucose.

Glucose-utilizing strains of *R. eutropha* and other bacteria have been intensively studied for biotechnological production of PHAs. To overcome the incapability of *R. eutropha* H16 to utilize glucose and to extent the substrate range, different attempts were undertaken. Historically, the first approaches were based on the isolation of spontaneous glucose-utilizing mutants (Franz et al., 2012; König & Schlegel, 1968), later also UV- (Schlegel & Gottschalk, 1965) and chemically (Kim et al., 1995) induced mutants were obtained. Glucose-utilizing mutants were provided by Schlegel and co-workers to ICI and one of them (deposited as *R. eutropha* NCIMB 11599) has been intensively exploited for the commercial production of PHA (Byrom, 1987; Chanprateep, 2010). The UV-induced glucose-utilizing mutants were able to transport glucose into the cells and exhibited constitutive glucose-6-phosphate-dehydrogenase activity (Schlegel & Gottschalk, 1965). Multiple independent spontaneous glucose-utilizing mutants were also isolated solely by provision of glucose to the media (Franz et al., 2012).

Recently, the glucose transport mechanism in G\textsuperscript{+1} mutant of *R. eutropha* H16 was resolved (Raberg et al., 2011, 2012). The glucose-utilizing G\textsuperscript{+1} mutant was generated in 1965 by Schlegel and Gottschalk by UV-mutagenesis (Schlegel & Gottschalk, 1965). A mutated and derepressed PTS\textsuperscript{Nag} was found to be responsible for the glucose transport (Raberg et al., 2011, 2012). A point mutation in nagR (a negative regulator of *nag* genes) of the G\textsuperscript{+1} mutant introduced a stop codon and caused a truncated repressor protein. Consequently, the derepression of the nag operon due to inactive NagR led to a constitutive overexpression of the nag operon in mutant G\textsuperscript{+1}. Furthermore, a missense Ala153Thr mutation in nagE was identified, which positively affected glucose uptake mediated by the mutated PTS\textsuperscript{Nag} in mutant G\textsuperscript{+1}. The *R. eutropha* H16 ΔnagRNagEA153Thr mutant strain showed the same phenotype as strain G\textsuperscript{+1} regarding glucose utilization (Raberg et al., 2012). Another independent study also identified mutations in nagR and nagE in glucose-utilizing *R. eutropha* NCIMB 11599 (Orita et al., 2012). The observed single mutation in nagR likewise inactivated the regulator, and an additional mutation Gly265Arg in NagE also improved glucose conversion. Although these two glucose-utilizing strains harbor different mutations in the nagE genes, they exhibit the same phenotype. The roles of these amino acids have to be studied. A further important finding is that the sugar is transported *via* facilitated diffusion and its transport is uncoupled from phosphorylation by PTS\textsuperscript{Nag}. Intracellular phosphorylation is mediated by glucokinase Glk (Raberg et al., 2012). Another interesting effect of a constitutively expressed and uncoupled PTS\textsuperscript{Nag} transporting sugar by facilitated diffusion is the avoidance of carbon catabolite repression (Contesse et al., 1969), allowing to use media harboring different carbon sources that can be utilized in parallel without diauxic growth (Raberg et al., 2012).

An alternative glucose-utilizing strain of *R. eutropha* was generated by metabolic engineering (Sichwart et al., 2011).
Heterologous expression of the energy-independent glucose-facilitated diffusion transporter, glf, from Zymomonas mobilis was used to mediate the glucose transport in the cell. Ralstonia eutropha H16 harboring glf was capable of growing with glucose as sole carbon source after a lag phase. Co-expression of glf and glk (glucokinase from E. coli) diminished the lag phase. The heterologous Glk compensated a possible metabolic bottleneck, possibly caused by insufficient expression/activity of the wild-type glucokinase. Reconstitution of glucose uptake and phosphorylation in R. eutropha via heterologous expression of glf and glk from Z. mobilis was recently confirmed (Orita et al., 2012). The strains harboring heterologous genes capable of glucose utilization stored comparable amounts of P(3HB) like the wild-type R. eutropha grown with gluconate (Sichwart et al., 2011).

Mannose
In contrast to glucose, no spontaneous mannose-utilizing mutants of R. eutropha occurred. Besides the lack of appropriate transporters for mannose, phosphorylation of mannose by the native hexokinase of R. eutropha was found to be less efficient than phosphorylation of fructose and glucose (Gottschalk, 1964). Moreover, mannose inhibits the oxidation of fructose in R. eutropha. An artificial pathway for the utilization of mannose by R. eutropha H16 was established (Sichwart et al., 2011). For this, mak and pmi genes, encoding mannofructokinase and phosphomannose isomerase from E. coli, were simultaneously expressed together with glf. Glf mediates not only the transport of glucose but also of mannose, while Mak phosphorylates mannose and Pmi converts mannose-6-phosphate to fructose-6-phosphate, which is metabolized in the ED-pathway. Ralstonia eutropha possesses an own gene encoding Pmi. However, moderate growth of the recombinant strains of R. eutropha, harboring either glf or glf together with mak, in the presence of mannose as sole carbon source, occurred only after prior cultivation on fructose (Sichwart et al., 2011). This effect was not observed if the cells were pre-cultivated in the presence of gluconate. Consequently, fructose seems to serve as inducer of pmi expression in R. eutropha. However, further investigations and corresponding genetic modifications must be achieved in order to exploit the native pmi from R. eutropha. Although phosphorylation of mannose is possible by a native hexokinase of R. eutropha (Gottschalk, 1964), the heterologous Mak improved the growth rate of R. eutropha significantly. P(3HB) contents of the recombinant strains grown on gluconate or mannose were similar, showing that mannose might be also utilized for industrial production (Sichwart et al., 2011).

Xylose and arabinose
Xylose and arabinose are sugars which can be derived from hemicelluloses (Figure S2). Ralstonia eutropha H16 is unable to utilize xylose or arabinose probably because it lacks enzymes mediating both, the uptake and the catabolism of these two sugars (Pohlmann et al., 2006). Uptake of xylose in E. coli is achieved by an ABC-transporter. The genome sequence did not reveal any hint for the presence of xylose-specific transporters in R. eutropha. In E. coli, the catabolism of xylose is initiated by xylose isomerase (XylA), which converts xylose to xylulose (Jeffries, 1983). Xylulose is then phosphorylated by xylulokinase (XylB) to xylulose-5-phosphate, which is further converted to ribulose-5-phosphate by ribulose-5-phosphate 3-epimerase (RPE, Table S2). In fungi and yeasts, catabolism of xylose is initiated by an NADP-dependent xylose dehydrogenase, which forms xylonolactone from xylose; then lactonase, xylonate dehydratase, 2-keto-3-deoxyxylonate dehydratase and aldehyde dehydrogenase convert the lactone via xylonic acid to 2-oxoglutarate (Jeffries, 1983). Ralstonia eutropha lacks almost all the necessary genes for xylose uptake and catabolism (Pohlmann et al., 2006). However, application of a xylose transporter (xylE), xylA and xylB from E. coli in addition to a putative R. eutropha’s genome encoded RPE was sufficient to establish xylose utilization in R. eutropha W50 (Liu et al., 2014).

Alternatively, R. eutropha grows on xylose-containing feedstocks in combination with Lactococcus lactis IO-1 by two-stage fermentation method (Tanaka et al., 1995). In the first stage, xylose was fermented to lactate and acetate by L. lactis and in the second stage PHA was synthesized by R. eutropha.

Ralstonia eutropha W50 was enabled to utilize arabinose via heterologous expression of a set of genes for L-arabinose uptake and metabolism from E. coli W3110 (Lu et al., 2013). The uptake of arabinose was catalyzed by the transporter which is encoded by the arafGH operon. In the cells L-arabinose was converted to L-ribulose by heterologous arabinose isomerase (AraA), then ribulose kinase (AraB) phosphorylated L-ribulose to L-ribulose-phosphate, which was finally transformed to D-xylulose-phosphate. The putative RPE of R. eutropha might be further involved in the metabolism of this carbohydrate. It was shown that the expression of the arabad genes in R. eutropha W50 was sufficient for utilization of L-arabinose; however co-expression of arafGH improved the uptake of L-arabinose (Lu et al., 2013).

Starch
Starch is one of the most abundant carbohydrates contained in plants (potatoes, wheat, corn, rice, etc.), and consequently appears in food wastes and starchy wastewaters (Figure S2; 127). The polysaccharide starch consists of glucose moieties linked by α1–4 glycosidic bonds. Amylases and glucoamylases hydrolyze this polysaccharide into maltose, maltotriose and glucose. Ralstonia eutropha lacks genes coding for putative amylases or glucoamylases. Therefore, the utilization of starch by R. eutropha is only possible after prior starch hydrolysis. Moreover, since glucose is the end product of starch saccharification, a glucose-utilizing strain of R. eutropha must be available (see above). There are at least two different approaches to transform starch into PHA. One method applies a two-step fermentation procedure (Yu, 2001). The starch wastes are converted by the active sludge containing acidogenic bacteria into volatile FAs (VFAs) such as formic, acetic, propionic and butyric acids. The VFAs could then be converted into bioplastic by PHA-producing bacteria like R. eutropha during the second fermentation step. Since propionic acid is available after the
first step, synthesis of P(3HB-co-3-hydroxyvalerate) is possible by this method. The second approach uses hydrolysis of polysaccharides into sugars by commercially available enzymes (Haas et al., 2008). Thereby, starchy wastes from a local potato chips factory were successfully converted to P(3HB) by glucose-utilizing R. eutropha NCIMB 11599 after previous enzymatic hydrolysis. However, the energy consumption during two-step fermentation, down-stream processing and saccharification cause additional costs negatively influencing the competitiveness of PHAs produced by this method. Therefore, the expression of heterologous amyloses could be another promising approach.

Lactose and galactose

Ralstonia eutropha H16 is not able to cleave the disaccharide lactose (Figure S1) or even to utilize galactose. Consequently, growth of the wild type on these sugars as a sole carbon source is not possible. Cleavage of lactose was conferred to the glucose-utilizing R. eutropha G+1 by heterologous expression of lacZ (β-galactosidase), lacI (inducer gene) and lacO (operator) from E. coli (Pries et al., 1990). However, utilization of galactose by R. eutropha remains unclear. One study showed that R. eutropha H16 was able to phosphorylate glucose and mannose, but not galactose, arabinose and sorbose (Gottschalk, 1964). Another study demonstrated that the recombinant glucose-utilizing strain R. eutropha G+1 excreted galactose into the medium, only if the lac genes were applied (Pries et al., 1990). A concomitant utilization of glucose and galactose was possible only if the gal genes were heterologously co-expressed with the lac genes. In contrast, recently another study demonstrated that the glucose-utilizing strain of R. eutropha DSM 545 (H1G+3) was able to grow on galactose, lactose or whey permeate as sole carbon sources harboring only the E. coli lac genes (Povolo et al., 2010). The lac genes were integrated in the phaZ (PHA-depolymerase)-gene to minimize the mobilization of P(3HB). Since strains G+1 and H1G+3 are UV-induced mutants, they might differ from the wild type and similar spontaneous mutants regarding the galactose metabolism. Therefore, it would be interesting to clarify the mechanisms of galactose uptake and utilization.

Utilization of lignin derivatives

Besides hemicellulose lignin is another abundant waste product derived from lignocelluloses (Figure S1). After chemical, physical or biological pretreatment of lignin, aromatic derivatives as p-coumaric, caffeic, feralic and sinapinic acid are released (Tomizawa et al., 2014). These substrates can be further metabolized by some bacteria (Pseudomonas putida, Sphingomonas paucimobilis) to oxaloacetate and pyruvate with the following intermediates: vanilllic, 4-hydroxybenzoic (4-HBA), 2,5-dihydroxybenzoic/genticis (2,5-DHBA), 3,4-dihydroxybenzoic/protocatechuic (3,4-DHBA), and 3,4,5-trihydroxybenzoic/gallic (3,4,5-TBHA) acids. Ralstonia eutropha H16 was able to utilize 4-HBA, 2,5-DHBA and 3,4-DHBA and the cells stored significant amounts of P(3HB) (Tomizawa et al., 2014). This can serve as a platform for establishment of the entire lignin degradation pathway in R. eutropha. The introduction of heterologous genes responsible for the conversion of lignin to 4-HBA, 2,5-DHBA and 3,4-DHBA should compensate the bottleneck in the lignin degradation pathway of R. eutropha.

Utilization of glycerol

Industrial production of biodiesel and plant oils is still developing; consequently, production of less valuable by-products is also increasing. Transesterification of oils releases waste rich in glycerol and free FAs (Figure S1; 109). As a main by-product of biodiesel industry, glycerol is an attractive cheap substrate for enhancing the competitiveness of PHAs and other biobased products (Posada et al., 2011).

Two glycerol kinases GlyK and two glycerol-3-phosphate dehydrogenases Gly3-DH are located on both chromosomes of R. eutropha (Table S2; Pohlmann et al., 2006). The genes from the first chromosome were found to be up-regulated, when R. eutropha H16 was grown on trioleate (Brigham et al., 2010). Glycerol enters the cells through passive diffusion and is phosphorylated by a kinase (Figure 1). Glycerol-3-phosphate is dehydrogenated to dihydroxyacetone-phosphate before it is metabolized in the sugar-degrading pathway.

Growth of R. eutropha on glycerol occurs at a low growth rate, which is partially due to high activity of hydrogenases overproducing reactive oxygen species, such as H2O2. Cells react with a stress response on oxidative stress grown on glycerol. The pattern of metabolic response during glycerol utilization does not differ significantly from autotrophic growth. The activity levels of hydrogenases, ribulose-1,5-bisphosphate-carboxylase-oxygenase (RuBisCO) and phosphoribulokinase (PRK) are comparable with autotrophic conditions (Friedrich et al., 1979; Schwartz et al., 2009).

Several attempts were undertaken to optimize glycerol utilization by R. eutropha strains DSM 545 and IPT 026. The glycerol and nitrogen contents in growth media were adjusted for P(3HB) synthesis (Campos et al., 2014; Cavalleiro et al., 2009). Concentrations of waste glycerol higher as 40 g L−1 were found to inhibit growth, probably due to impurities in the feedstock (Cavalleiro et al., 2009). Besides P(3HB), synthesis of copolymers P(3HB-co-3-hydroxyvalerate) and P(3HB-co-4-hydroxybutyrate-co-3-hydroxyvalerate) has been established from by-products of biodiesel production (Cavalleiro et al., 2012; García et al., 2013). However, to significantly accelerate the growth rate it was not sufficient to optimize only the growth conditions. A metabolic network of intracellular processes during growth on glycerol was analyzed with elementary flux modes and yield space analysis, and putative links between central metabolism and P(3HB) synthesis were marked (Lopar et al., 2014). A linkage between Gly3-DH’s cofactor and NAD(H)/NADP(H) transhydrogenases was indicated. Facilitation of crude glycerol uptake and improving the function of GlyK and Gly3-DH and their interaction with the anabolic EMP-pathway and its metabolites was suggested (Lopar et al., 2014). Indeed, integration of glpKEc alone or together with aquaglyceroporin (glpF Ec) in the R. eutropha H16 genome compensated the slow growth rate and significantly increased the P(3HB) productivity (Fukui et al., 2014).
Another study demonstrated that the presence of glucose in the medium even at low concentrations reduces the consumption of glycerol in *R. eutropha* DSM 545 (Špoljarić et al., 2013). Probably, the high intracellular pool of glycerol-3-phosphate and dihydroxyacetone-phosphate serves as a negative feed-back controller of glycerol consumption. These data are also in accordance with an assumption about inhibition of initial glycerol activation reactions by the products of the EMP-pathway (Lopar et al., 2014).

Another issue of interest is the molecular weight of P(3HB) derived from glycerol. An unspecific incorporation of glycerol by PhaC can lead to a polymer chain termination during prolonged glycerol cultivation (Tanadchangsaeng & Yu, 2012). Several studies reported on the reduction of the molecular weight of polymers produced from glycerol (Cavalheiro et al., 2009; Tanadchangsaeng & Yu, 2012). Since no significant difference in molecular weights of PHAs produced from glycerol or fructose were observed in *R. eutropha* H16 with facilitated glycerol metabolism (glyKec and glpFec), the reduction of intracellular glycerol concentration probably reduces the frequency of chain terminations (Fukui et al., 2014).

### Utilization of diverse organic acids

#### Fatty acids

Since FAs can be transformed into PHAs with a very high yield they are promising substrates for PHA production and have the potential to reduce production costs. In contrast to carbohydrates, a variety of organic acids are taken up by *R. eutropha* H16, so here is no need for substrate range expansions. However, optimization of the conversion of FAs to products has to be achieved. Organic acids, such as acetic, propionic and succinic acids, are fermentation products and common intermediates (in CoA-thioester form) of the central metabolism in *R. eutropha*. Acetic and succinic acid are oxidized in the TCC, while propionyl-CoA is oxidized in the methylcitrate cycle (MCC). Acyl-CoA thioesters of butyric and valeric acid (VA) as well as MCL- or long-chain-length FAs first undergo β-oxidation to yield acetyl-CoA (Figure 1).

Since acetyl-CoA can be completely oxidized in TCC, growth on the above mentioned substrates yielding acetyl-CoA is combined with a loss of carbon as CO₂ and a lack of three-carbon (C3) and four-carbon (C4) units in the central metabolism. The anaplerotic glyoxylate pathway, which bypasses the decarboxylation steps of the TCC, mediates the conversion of the acetic residue (C2) to C4-compounds. The glyoxylate pathway comprises cleavage of isocitrate into succinate and glyoxylate; the latter is further condensed with acetyl-CoA by malate synthase to yield acetyl-CoA (Figure 1). The concomitant product of the glyoxylate bypass, succinate, is metabolized in the TCC. Another anaplerotic pathway mediates between C3- and C4-compounds during growth on C4-organic acids like succinate or malate (Schobert & Bowien, 1984). The interconversion of C4- and C3-compounds is catalyzed by PEP-carboxykinase to yield PEP from oxaloacetate by decarboxylation (Utter & Kolenbrander, 1972). Alternatively, decarboxylation of malate/oxaloacetate catalyzed by malic enzyme or oxaloacetate decarboxylase yields pyruvate (Figure 1; Bruland et al., 2010). Thus, C4-compounds like malate and oxaloacetate are transformed to C3-products like pyruvate and PEP for gluconeogenesis. Decarboxylation of malate to pyruvate is a NADPH-dependent reaction, and thus the C3/C4 metabolism regenerates the main cofactor of P(3HB) synthesis (Bruland et al., 2010; Yu & Si, 2004).

Acidifying agents commonly occur in the natural environment of *R. eutropha*, and even moderate concentrations of them might lower pH and inhibit cell growth. SCL-FAs, like acetic, propionic, butyric and VA, are often referred to as VFAs. They can freely diffuse through the membrane, acidify the cytoplasm which causes the toxic effect and consequently slows down metabolite production. Dissociated FAs reduce the proton gradient through the membrane, increase osmotic pressure and thus interfere with efficient energy metabolism (Wang et al., 2010). To overcome the toxic effect of FAs, *R. eutropha* possesses a detoxification mechanism (Lee et al., 2006, 2009). Besides the toxicity, pure-free FAs are relatively expensive. Therefore, the controlled co-feeding at low concentrations in the medium is the prevailing method (Steinbüchel & Lütke-Eversloh, 2003).

#### Even chain FAs

Even chain FAs provide exclusively 3HB-monomers for PHA synthesis in *R. eutropha* H16. Among all acids utilized by *R. eutropha*, acetic acid exhibits the second highest dissociation constant and consequently causes severe toxic effects. The detoxification mechanism comprises the rapid metabolism of acetic acid. The tolerance of *R. eutropha* towards acetic acid increases with the cell mass concentration and concomitant increase in utilization rate of acetic acid (Yu & Wang, 2001). Therefore, acetate kinase and/or acetyl-CoA synthase activate(s) the acetic-residue with CoA. Acetyl-CoA is further directed to P(3HB) synthesis, TCC and other biosynthetic pathways, such as lipid or amino acid synthesis. Carbon flux analysis showed that the majority of the consumed acetic acid is divided between P(3HB) synthesis and TCC and only a small part is condensed with glyoxylate (Yu & Si, 2004). Growth of *R. eutropha* on acetic acid is associated with the upregulation of such detoxifying enzymes as, for example, catalases, which are involved in a defensive response of *R. eutropha* on the toxic effect of organic acids (Lee et al., 2009). In a mixture of organic acids, acetic acid is consumed after other acids like propionic, lactic or butyric acid, are exhausted (Yan et al., 2003). Furthermore, two acetic-residues are necessary to produce one molecule of 3HB. If butyric acid is used as sole carbon source, one molecule of butyric acid provides one molecule of 3HB or two molecules acetyl-CoA, which are then oxidized in TCC, resulting in better cell growth and P(3HB) yield. In addition, butyric acid is consumed to a higher rate and less toxic to the cell (Yan et al., 2003, Yang et al., 2010). Recently, it was shown that butyric acid serves also as precursor for the production of PHAs with C6-units as described below (Jeon et al., 2014).

γ-Butyrolactone, 4-hydroxybutyric acid (4HB), 4-chlorobutyric acid and 1,4-butanediol are precursors leading to incorporation of 4HB in PHAs. *Ralstonia eutropha* synthesizes P(3HB-co-4HB) copolymers with different
4HB-fractions depending on the carbon source (Kunioka et al., 1989). However, although these substrates are taken up by the cells, partially degraded and incorporated into the copolymer, R. eutropha H16 is not able to grow with 4HB or 1,4-butanediol as sole carbon source (Valentini et al., 1995). γ-Butyrolactone is putatively hydrolyzed, 4-chlorobutyric acid is dechlorinated and 1,4-butanediol is oxidized to 4-hydroxybutyl-CoA, and the latter is then incorporated into the copolymer (Kunioka et al., 1989). It is assumed that intracellular degradation of 4HB is carried out via succinate semialdehyde to succinate (Valentini et al., 1995). P(3HB-co-4HB) exhibits biocompatibility, decreased crystallinity and consequently better flexible/elastic properties as P(3HB) and is degraded by lipases and esterases (Jaeger et al., 1995).

Optimization of 4HB uptake in R. eutropha improves conversion of 4HB to acetyl-CoA and its availability for the central metabolism, while the molar 4HB fraction in the copolymer decreases (Steinbüchner et al., 1994). Synthesis of P(4HB) homopolymer was possible by P(3HB)-leaky mutants of R. eutropha JMP222, harboring multiple copies of heterologous PHA biosynthesis genes from R. eutropha H16, if 4HB was used as the carbon source (Steinbüchner et al., 1994).

However, the 4HB-precursors are toxic for cells at high concentrations and costs, that is why the two-step batch or controlled co-feeding are the most commonly used procedures. The combination of sugar- or FA-containing cheap substrates as main carbon source with more expensive co-substrates is more beneficial for industrial production. Soybean, spent palm oil or waste glycerol in combination with 4HB-precursor such as γ-butyrolactone were successfully applied for production of P(3HB-co-4HB). 4HB fractions of 6–10 mol% in P(3HB-co-4HB) were achieved by co-feeding soybean oil and 0.5–1% (w/v) γ-butyrolactone (Park & Kim, 2011) or even with up to 15 mol% with spent palm oil plus 0.5% (w/v) γ-butyrolactone (Rao et al., 2010). Successful variation of 4HB-content in P(3HB-co-4HB) copolymers synthesized by R. eutropha DSM 545 from waste glycerol supplemented with γ-butyrolactone was demonstrated (Cavalheiro et al., 2012). At higher levels of dissolved oxygen, more PHA, though with minor 4HB-fraction, was stored and vice versa. The molar composition and in particular the amount of 4HB in the copolymer can be also manipulated applying alkanoates as co-substrates. This effect was observed with propionic, butyric, valeric and hexanoic acids; however, with propionic acid the highest molar content of 4HB was detected (Kimura et al., 1999). The presence of propionic acid inhibited the degradation of 4HB, and up to 43 mol% of 4HB were incorporated in the copolymer (Cavalheiro et al., 2012; Kimura et al., 1999). Besides, the co-utilization of γ-butyrolactone and propionic acid led to the synthesis of a new P(3HB-co-4HB-co-3HV) terpolymer. P(4HB) homopolymer could be purified via fractionated isolation of PHA from R. eutropha grown on mixture of 4HB and propionic acid (Kimura et al., 1999). For further details of 4HB-containing copolymers see below.

After it was shown that PhaCRe is also able to utilize MCL-hydroxyalkanoates (Dennis et al., 1998), the production of copolymers with chain lengths >C5 has been studied intensively in R. eutropha. It is known that supply of MCL-precursors is hindered by highly active β-oxidation. Precursors of MCL-PHAs are CoA-thioesters of FAs that are usually completely degraded to acetyl-CoA. Two operons were found to be responsible for FA degradation in R. eutropha (Table S2; Brigham et al., 2010). The FA degradation operons seem to be constitutively expressed on fructose and upregulated in presence of FAs (Shimizu et al., 2013). Consequently, high amounts of 3HB-precursors are provided by highly active β-oxidation and abundance of acetyl-CoA. Chemical inhibition of β-oxidation by acrylate resulted, in contrast, in the integration of 3-hydroxyhexanoate (3HHx) and 3-hydroxyoctanoate in copolymers by R. eutropha (Green et al., 2002). Molecular engineering techniques and appropriate carbon sources optimized P(3HB-co-3HHx) production in R. eutropha (Budde et al., 2011; Mifune et al., 2010). Therefore, the efflux of β-oxidation intermediates to PHA synthesis was forced via overexpression of an (R)-enoyl-CoA hydratase or a 3-hydroxyacyl-ACP:CoA transferase genes. Manipulation of the phaCAB operon resulted in copolymers with new compositions (Matsumoto et al., 2001; Steinbüchner & Lüte-Eversloh, 2003). Two homologues of a (S)-specific 2-enoyl-CoA hydratase/(S)-3-hydroxyacyl-CoA dehydrogenase from the β-oxidation operons were deleted in order to improve the incorporation of MCL-hydroxyalkanoates (Insomphun et al., 2014). Alternatively, P(3HB-co-3HHx) was successfully produced from butyric acid as single carbon source (Jeon et al., 2014). For this, phaCRe was replaced by phaC from Rhodococcus aetherivorans I24 exhibiting broad substrate specificity, and all three genes encoding for acetoacetyl-CoA reductases were deleted in R. eutropha. In this mutant, the 3HB-content in the copolymer was reduced. Condensation of an acetic-residue with butyric acid provided as carbon source yielded 2-ketohexanoyl-CoA, which was thereafter incorporated into the copolymer. The C2- and C4-CoA thioesters are putatively condensed by one of the multiple β-ketohiolases of R. eutropha. However, BktB is the most suitable candidate, since it assisted in this reaction in an artificial 1-hexanol pathway (Dekishima et al., 2011). Further approaches for MCL-PHA biosynthesis based on FA metabolism are discussed in more detail elsewhere (Riedel et al., 2014).

Odd chain FAs

Usually odd chain FAs are degraded via β-oxidation to acetyl-CoA and propionyl-CoA as end products. Propionyl-CoA contributes to synthesis of a heteropolymer consisting exclusively of 3HB and 3-hydroxyvaleric acid (3HV), i.e. P(3HB-co-3HV) (Doi et al., 1988). Different 3HB/3HV ratios influence physical properties of synthesized copolymers. Generally, the technical applications of P(3HB-co-3HV) are more beneficial than P(3HB) due to improved flexibility and reduced crystallinity. Melting temperature of these copolymers decreases with increased 3HV amounts (Wang et al., 2013).

Once propionyl-CoA is formed from propionic acid, it can be directed to the central metabolism or to PHA metabolism (Lee et al., 2009). The catabolism of propionic acid in MCC yields succinate and pyruvate with the latter further decarboxylated to acetyl-CoA (Figure 1, 5). Serving as sole
carbon source, propionic acid is mainly converted to acetyl-CoA, resulting in a restricted flux of propionyl-CoA to the copolyester synthesis. Interestingly, another β-ketothiolase, BktB, is responsible for P(3HB-co-3HV) synthesis. Whereas BktB is able to condense propionyl-CoA and acetyl-CoA, PhaA is not (Slater et al., 1998). *Ralstonia eutropha* lacking bktB grows as the wild type on propionic acid, but is impaired in synthesis of 3HV from propionyl-CoA (Lindenkamp et al., 2012). Thus, the copolymer from the bktB-lacking mutant contained only up to 3.5 mol% of 3HV.

Propionyl-CoA is condensed by BktB with acetyl-CoA, and reduced by PhaB to 3HV-CoA. If the ratio of acetyl-CoA/propionyl-CoA in the cell is high, more 3HB-units are incorporated into the copolymer. In contrast, if the concentration of propionic acid in the medium is increased, this shifts this ratio towards propionyl-CoA, and higher 3HV-molar fractions in the copolymer (22–45 mol%) (Doi et al., 1987). When acetic and propionic acids are present in the culture medium, acetyl-CoA is obtained from both substrates (Doi et al., 1987), but it was shown that propionic acid is more favorably consumed as acetic acid (Yang et al., 2010). Metabolic fluxes show that only small amounts of propionyl-CoA are converted to 3HV, while MCC consumes the majority of propionyl-CoA (Yu & Si, 2004). Consequently, the acetyl-CoA/propionyl-CoA ratio in the cell is higher only if propionic acid is provided, and the copolymer contained 2–28 mol% of 3HV (Doi et al., 1987). In general, the application of a mixture of acids allows the modulation of PHA synthesis yielding the desired copolymer composition (Yang et al., 2010).

Provision of butyric (Yu & Si, 2004) or oleic (Marangoni et al., 2000) additionally to propionic acid improves the total PHA yield. However, only due to an increasing acetyl-CoA/propionyl-CoA ratio and hence higher 3HB supply, while total 3HV content is less affected. An interesting effect on carbon source, valeryl-CoA is degraded via β-oxidation to acetyl-CoA and propionyl-CoA. Simultaneous application of butyric acid and VA reduces 3HV molar content due to higher 3HB content (Doi et al., 1988). BktB plays also a significant role in metabolism of VA. *Ralstonia eutropha* lacking bktB retains the capability to grow on VA and on propionic acid, but incorporates higher amounts of 3HV (up to 97 mol%) into the copolymer (Lindenkamp et al., 2012).

Propionic and VAs are not applicable in high concentrations, because of their toxicity and costs. Utilization of diverse cheap feedstocks supplemented with propionic or VA are more suitable for P(3HB-co-3HV) synthesis. Up to 90% PHA of CDW with 7 mol% of 3HV was obtained from palm kernel oil with propionic acid as co-substrate or 89% PHA of CDW with 14 mol% of 3HV from olive oil supplemented with VA (Lee et al., 2008). Generally, higher 3HV fractions from VA in comparison to propionic acid are explained due to sufficient provision of acetyl-CoA for the central metabolism from degradation of plant oils. Thus, less VA is cleaved and directed in the central metabolism, but enhanced integration of 3HV into the copolymer occurs.

Levulinic acid (LA) serves as sole carbon and energy source and as precursor for 3HV and 4HV for *R. eutropha*. It appears in waste products of wood industry and can be derived from acidic hydrolysis of starch and lignocellulosic biomass (Assary et al., 2010). LA is the oxidized form of 4HV, and its catabolism starts with activation to levulinyl-CoA by a membrane-bound acyl-CoA synthetase. The acyl-CoA synthetase activity was absent in cells grown on acetate, demonstrating an inducible character of LA conversion (Jaremko & Yu, 2011). Acyl-CoA dehydrogenase and enoyl-CoA hydratase/carnithine racemase were also induced in the presence of LA, together with glutathione transferase and a chaperon, which might be involved in a detoxifying response (Brämer, 2002). Acyl-CoA dehydrogenase and enoyl-CoA hydratase/carnithine racemase activity support the assumption that LA is degraded via β-oxidation. Thus, the expected end products of the catabolism of LA in *R. eutropha* are acetyl-CoA and propionyl-CoA. Since the β-oxidation inhibitor acrylate prevented growth of *R. eutropha* with LA as sole carbon source, thiolytic cleavage of CoA-derivatives of LA was indirectly approved (Brämer, 2002). However, no other intermediates, except acetyl-CoA and propionyl-CoA were observed in in vitro enzyme assays, supporting the assumption that levulinyl-CoA is directly cleaved into acetyl-CoA and propionyl-CoA (Jaremko & Yu, 2011).

Since the utilization of LA as sole carbon source yields low values of biomass and PHA content, co-feeding of LA with glucose or fructose increases the productivity. When *R. eutropha* was grown under optimized conditions with glucose and LA, cells reached higher cell densities and stored PHA up to 81% of CDW (Wang et al., 2013). The 3HV monomer composition varied from 25 to 54 mol%. It was shown that at low LA-concentrations, it was utilized at higher rates as glucose and fructose. Concerning PHA synthesis, 3HV-incorporation was less affected by nitrogen limitation, as it is known for the 3HB-monomer. The highest 3HV fraction in copolymers was observed in the transition growth phase, when nitrogen was still available in excess and before significant 3HB synthesis started. Thus, due to the variation of C/N-ratio the desired PHA composition can be achieved (Jaremko & Yu, 2011). During growth on LA, different monomers, such as 3HV and 4HV, can be incorporated into the copolymer (Valentin & Steinbüchel, 1995). Furthermore, recombinant *R. eutropha* was able to synthesize terpolymers consisting of 3HB, 3HV and minor amounts of 4HV when cultivated with 4HV or 4-valerolactone (Valentin et al., 1992).

Application of 5-chlorovaleric acid alone or in combination with VA as the carbon source resulted in the synthesis of P(3HB-co-3HV-co-5-hydroxyvalerate) (Doi et al., 1987). Further substrates, such as 5-hydroxyvalerate and 5-pentadeic acid, were successfully used for synthesis of P(3HB-co-3-hydroxypropionate-co-5-hydroxyvalerate) yielding 5-hydroxyvalerate contents up to 10 mol% (Chua et al., 2013). The PHA-negative mutant *R. eutropha* PHB 4 harboring phaCRe or mutated copies of phaCRe, was cultivated in a.
two-step fermentation. In the second step, different concentrations of 5-hydroxyvalerate or \( \omega \)-pentadecalacton were tested. The obtained lipase-degradable copolymer exhibited reduced crystallinity, melting temperature and tensile strengths, and increased elongations to break-values in comparison to P(3HB). Alternatively, when other odd chain compounds like 3-hydroxypropionate (3HP), 1,5-pentanediol or 1,7-heptanediol were used as carbon source, synthesis of P(3HB-co-3HP) with a 3HP-fraction up to 7 mol% was observed. From these, 1,5-pentanediol led to the highest copolymer content (Nakamura et al., 1991). Copolymers containing 3HP up to 2.1 mol% could be also produced from structurally unrelated substrates via modified metabolism of *R. eutropha* (Fukui et al., 2009). Heterologous malonyl-CoA reductase and the 3HP-CoA synthetase domain of trifunctional propionyl-CoA synthase from CO\(_2\)-fixation pathway of *Chloroflexus aurantiacus* were introduced in *R. eutropha* and catalyzed 3HP-formation from acetyl-CoA via malonyl-CoA, when the cells were grown with fructose or even chain alkanoates.

**Amino acids and modifications on amino acid metabolism**

*Ralstonia eutropha* H16 synthesizes PHAs also from amino acids as sole carbon source although high yield of PHAs were not obtained, since the dissimilation of amino acids is associated with nitrogen supply (Kimura et al., 2003). Moreover, single amino acids are costly. Grown on amino acids, *R. eutropha* accumulates either P(3HB) or P(3HB-co-3HV). 3HV is provided from propionyl-CoA obtained during degradation of leucine, isoleucine, threonine and valine (Figure S2). L-Valine was studied in more detail, since it was more effective regarding P(3HB-co-3HV), yielding the copolymer with 10 mol% of 3HV.

Since propionyl-CoA is involved in the metabolism of some amino acids, an auxotrophic mutant of *R. eutropha* H16 with altered anabolism of branched-chain amino synthesized P(3HB-co-3HV) from single unrelated substrates such as fructose or gluconate. The mutant excreted valine, leucine and isoleucine into the medium, when a nitrogen source was available in excess, while ammonium limitation yielded P(3HB-co-3HV) in propionic acid-free medium (Steinbüchel & Pieper, 1992). Such intracellular overproduction of the amino acids or related intermediates provides propionyl-CoA from renewable resources.

Recombinant *R. eutropha* PHB-4 harboring phaC from *Pseudomonas* sp. 61-3 synthesized P(3HB-co-3-hydroxy-4-methylvalerate) [P(3HB-co-3H4MV)] on fructose medium supplemented with leucine (Saika et al., 2011). Amino acid co-feeding was chosen as an alternative to supply of costly 4-methylvalerate, since the leucine backbone is identical with 3H4MV (Figure S2). To increase the 3H4MV fraction without leucine co-feeding, the end product feedback inhibition was overcome by mutagenesis, and an increased metabolic flux to leucine biosynthesis was obtained. Consequently, the mutation strain incorporated higher amounts of 3H4MV when grown on fructose.

L-Serine, L-alanine and L-threonine supplemented in addition to \( \beta \)-oxidation-unrelated substrates (acetic acid, glucose) led to P(3HB-co-4HB) accumulation (Kimura et al., 2008).

Supplementation of l-amino acids had a positive effect on CDW with highest values by l-threonine and threefold higher P(3HB-co-4HB) yields, as shown in the absence of amino acids. In presence of L-alanine and L-serine higher 4HB-fractions than with L-threonine occurred. This is putatively due to more favorable utilization of L-threonine for cell growth and 3HB synthesis.

**Mercaptoalkanoic acids**

Cultivated in presence of sulfur-compounds, such as 3-mercaptopropionic (3MP), 3,3′-thiodipropionic (TDP), 3,3′-dithiopropionic (DTDP), 3-mercaptoputryic (3MB) or 3-mercaptopvaleric (3MV) acid, *R. eutropha* incorporates 3MP, 3MB or 3MV into the corresponding copolymers with 3HB (Lütke-Eversloh et al., 2001; Lütke-Eversloh & Steinbüchel, 2003). However, *R. eutropha* cannot utilize these substrates as the sole carbon source. The enzymes mediating uptake of 3MP, TDP and DTDP or cleavage of TDP and DTDP are important for improving the availability of 3MP for polythioester (PTE) synthesis. In contrast, the activity of enzymes metabolizing 3MP to propionyl-CoA are less favorable, since they compete with PTE synthesis for 3MP. PTEs were first identified in 2001 (Lütke-Eversloh et al., 2001). The copolymers of 3HB and 3-mercaptopalkanoates exhibit biotechnologically attractive features differing from PHAs in their melting point or glass transition temperature. Moreover, in contrast to PHAs, PTEs are not biodegradable (Elbanna et al., 2004).

The metabolism of 3MP, TDP and DTDP in *R. eutropha* is still not completely clear. Genome-wide transcriptome analysis of *R. eutropha* H16 revealed genes which were upregulated and might participate in the utilization of TDP or DTDP (Peplinski et al., 2010). Consequently, several deletion mutants were generated to identify genes required for the metabolism of these substrates (Doberstein et al., 2014). Single and double deletion mutants lacking ABC-type transporter(s) exhibited slower growth with DTDP (Table S2). Interestingly, only a deletion of the cluster H16_A0357-A0359 led to significantly lower 3MP-fractions in the copolymer and only during growth with DTDP. In contrast, double deletion ΔH16_A0357-A0359A16_A368-A3660 resulted in higher 3MP-fractions. Applying DTDP as precursor, less 3MP was also incorporated if bug genes encoding putative *Bordetella*-type uptake proteins were deleted. DTDP is probably metabolized by a disulfide reductase to 3MP, while TDP is converted by a sulfide hydrolase to 3MP with concomitant 3HP release. Disulfide interchange proteins DsbA, DsbD, FrnE, rhodanase-related sulfurtransferase and also hydrolase S-adenosylhomocysteinase, might be involved in the cleavage of DTDP since the expression of the corresponding genes was upregulated in presence of sulfur-compounds (Peplinski et al., 2010). However, only deletion of *dsbD* led to a significant reduction of 3MP-molar fraction in the copolymer (Doberstein et al., 2014).

3MP is further activated to 3MP-CoA and polymerized by PhaC. PhaB3 seems also to be important for P(3HB-co-3MP) synthesis, since its expression was two- to five-fold higher than during growth on gluconate (Peplinski et al., 2010). On the other hand, the expression of *phaB3* was reduced upon the
onset of significant P(3HB) synthesis (Budde et al., 2010). Genes of FA metabolism were also upregulated during growth with DTDP or TDP, suggesting the metabolism of 3MP and 3-hydroxypropionate to propionyl-CoA (Table S2; Peplinski et al., 2010). Other genes, encoding cysteine dioxygenase (CdoA), 2-methylcitrate synthase (PrpC1) and propionate CoA transferase (Pct), of *R. eutropha* were not differentially expressed in the microarray analysis. However, these enzymes have also been studied in terms of PTE synthesis. CdoA converts 3MP to 3-sulfonpropionate (Bruland et al., 2009). 3-Sulfonpropionate can be converted to propionyl-CoA and oxidized in the MCC. The activity of the key MCC-enzyme, PrpC1, was increased in the late stationary phase in presence of 3MP-precursors (Peplinski et al., 2010). The lack of *pct* in the corresponding negative mutant of *R. eutropha* did not affect 3MP fraction in the copolymer (Lindenkamp et al., 2013). The gene might be silent but the enzyme was able to activate 3MP with CoA, which suggests that overexpression of *pct* might increase the 3MP fraction in the copolymer.

Since synthesis of P(3MP) homopolythioesters was shown only for *E. coli*, but not for *R. eutropha*, suppression of 3HB-CoA synthesis and deletion of β-ketothiolases was investigated (Lindenkamp et al., 2010). Deletion of 9 of 14 β-ketothiolases in fact resulted in the increased molar 3MP-fraction in the copolymer due to lowered 3HB-contents.

Thus, it was shown that the 3MP-fraction in the copolymer synthesized by *R. eutropha* can be varied. The most important drawbacks like low yield copolymer synthesis and costliness of the precursors need to be addressed. Further improvements by metabolic engineering and establishment of copolymer synthesis from inorganic sulfur as PTE-precursor would be advantageous (Wübbeler & Steinbüchel, 2014).

**Utilization of C1-compounds**

Among biotechnologically relevant C1-substrates such as CO, CO₂, methanol and formate, only CO₂ and formate are utilized by *R. eutropha* as the sole carbon source (Friedrich et al., 1979). CO₂ and formate differ from other substrates discussed in this study, since they are metabolized in a similar manner via the CBB cycle.

In the presence of CO₂ as the sole carbon and H₂ as energy source, *R. eutropha* assimilates CO₂ by the key enzyme RuBisCO (Figure 1), while three distinct oxygen-tolerant [NiFe]-hydrogenases deliver energy via H₂ oxidation (Lenz et al., 2010). One hydrogenase is membrane-bound and channels electrons into the electron transport chain. The second hydrogenase is soluble and generates reducing equivalents (NADH), which are especially needed in the absence of organic compounds for CO₂-fixation. The third regulatory hydrogenase controls gene expression of the membrane-bound and soluble hydrogenases in response to the availability of H₂ (Burgdorf et al., 2005; Kleihues et al., 2000).

If formate serves as a substrate, hydrogenases and enzymes of CBB cycle are highly active (Friedrich et al., 1979). The activities of two formate dehydrogenases are also increased. These enzymes oxidize formate to CO₂, which is then fixed in CBB cycle. As formic acid is very toxic, it is not applicable at high concentrations, as aldehyde it may inhibit enzymes of the central metabolism. In general, it was shown that the cells reduce the amino acid biosynthesis (Shikimate pathway), pyrimidine and purine formation, while the P(3HB) synthesis enzymes demonstrate higher activity in presence of formate (Lee et al., 2006). Due to its toxicity, low energy potential and low PHA/biomass yield, formate is not suitable as sole carbon source. However, like other VFAs it is a common ingredient of carbohydrate hydrolysates. Moreover, since formic acid can be electrochemically generated from CO₂ and water, it can be further microbiologically converted to diverse products. Electricity generated from regenerative energies sources can be stored in form of electromicrobial fuels. Recombinant strains of *R. eutropha* were used electromicrobial conversion of CO₂/formate to higher alcohols (Jeon et al., 2013; Li et al., 2012). These auto- and mixotrophical approaches comprise, on one hand, the possibility to store energy and on the other, consume CO₂, which is also beneficial for the environment.

**Conclusions**

In summary, the metabolic versatility of *R. eutropha* has made this bacterium to a model production strain for PHA metabolism in the past. The understanding of its metabolism based on the availability of the genome sequence and a range of transcriptomic, proteomic and metabolomic data, gene-deletion vectors and expression systems recently broadened the spectrum of applications of *R. eutropha* as a production platform. Thus, the potential for high-yield production of diverse tailor-made biopolymers by *R. eutropha* H16 has been demonstrated. The application of metabolically engineered strains of *R. eutropha* revealed a potential of this bacterium as a platform for large-scale biotechnological production of various chemicals and offers an alternative for chemical synthesis. Various ways to extend and optimize the conversion of inexpensive renewable feedstocks have been established. The autotrophic production of bio-based products is another important separate topic and was only briefly mentioned in this review. However, production of valuable products, derived from the P(3HB) or central metabolism, applying CO₂ as carbon source has been intensively studied. In parallel to chemical synthesis and the application of other production strains, synthesis of additional chemicals like organic acids by *R. eutropha* seems to be promising and should be established in future to replenish the range of synthesized bioproducts. The chemical production of organic acids (acetic, succinic or pyruvic acid) may be replaced by sustainable microbial production. The versatile metabolism of *R. eutropha* can be successfully modulated based on research experience of more than 50 years. Thus, simple, renewable and inexpensive feedstocks can be used by *R. eutropha*. The pathway's bottlenecks can be identified and reconstructed, undesired metabolic fluxes and by-products can be reduced or eliminated based on the advances in genome sequencing, functional genomics, transcriptomics, proteomics and metabolomics.

**Declaration of interest**

The authors report no declarations of interest. The authors alone are responsible for the content and writing of this article.
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