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REVIEW

Genome characteristics dictate poly-R-(3)-hydroxyalkanoate production in *Cupriavidus necator* H16

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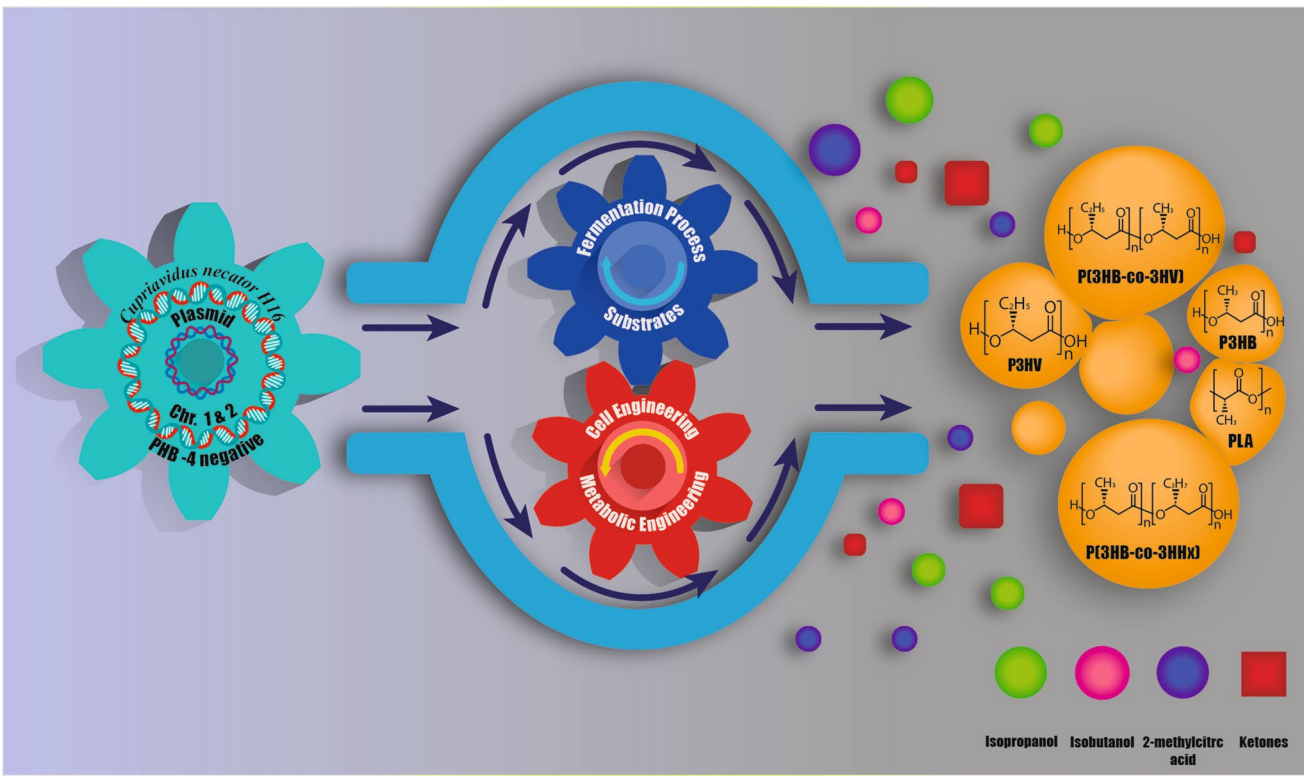
Abstract

Cupriavidus necator H16 is a well-recognized enterprise with efficient manufacturing machineries to produce diverse polymers belonging to polyhydroxyalkanoates (PHAs) family. The genome fingerprints, including PHA machinery proteins and fatty acid metabolism, had educated engineering strategies to enhance PHAs production. This outstanding progress has enlightened us to present an exhaustive examination of the ongoing research, addressing the great potential design of genome features towards PHA production and furthermore, we show how those acquired knowledge have been explored in other biotechnological applications. This updated-review concludes that the combination of an optimal strain selection, suitable metabolic engineering and a large-scale fermentation on oil substrates is critical to endow the ability of incorporating mcl-PHAs monomers in this organism.

Graphical Abstract



Keywords PHA synthase · Phasins · Metabolic engineering · Medium chain length · Mutants · Biofuels

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Introduction

Microbial cells are highly equipped with specialized genetic components in their genome to produce numerous value-added products of biotechnological applications. A group of biodegradable polyesters belonging to polyhydroxyalkanoates (PHAs) are one among them. PHAs are naturally synthesized by diverse prokaryotic organisms under nutrient limitation and stress conditions as carbon reserve storage material (Chen 2009). They are majorly made up of linear chain of 3-hydroxyacyl CoA (3-HA) monomers, in which, 3-HA monomers can be either short chain length (scl; C3–C5), medium chain length (mcl; C6–C12) or long chain length (> C12). PHA synthase, the key PHA enzyme, catalyses the polymerization of 3-HA monomers resulting in the formation of cytoplasmic granules (Rehm 2003). The recovered cytoplasmic PHAs have commercial value offering a range of applications include bioplastics, drug delivery system, tissue engineering etc (Chen 2009).

Polyhydroxybutyrate (PHB; C4) is the most common natural homo-polymer in PHAs family, as almost all reported prokaryotic genera, except few *Pseudomonas* species, produce and reserve it (Madison and Huisman 1999). PHB, being brittle and less elastic, has very little potential compared to copolymers [e.g., Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)], discovered later through the coupled efforts of genetic engineering and fermentation studies (Madison and Huisman 1999; Chen 2009). The copolymers of mcl-PHAs exhibit sticky, elastomeric properties and the production of high quality-end PHAs with improved thermo-mechanical properties had become one of the major growing areas in this booming field. In this regard, for past several decades, organisms like *Cupriavidus necator* H16, *Azotobacter vinelandii, P. putida, Synechocystis* sp. PCC 6803 have been extensively employed as model platforms for obtaining PHAs (Madison and Huisman 1999). Particularly, *C. necator* H16, belonging to the genus *Cupriavidus* and capable of storing up to 90% PHB of its cell dry weight, have received a lot of attention and been subjected to profound investigations for long time (Fig. 1). The genome of this organism is complete with two chromosomes (4.05 and 2.91 Mb) and a plasmid (pHG1; 0.4 Mb) (Schwartz et al. 2003; Pohlmann et al. 2006). The recent transcriptional, proteomic and metabolic profiling analysis have uncovered significant information related to PHA production (Brigham et al. 2012; Fukui et al. 2013; Shimizu et al. 2013). At the same time, the advent of molecular techniques and bioinformatics have identified the role of various metabolic pathway proteins. Since then, this organism has experienced many major advances in engineering pathways and have undergone modifications to enhance the polymer production. It is evident that the degree of publications had shown drastic increase for *C. necator* H16. In spite of these great efforts, researchers have often end-up with very limited mcl-PHA monomers in the polymer, which is not quite effective but challenging for future investigations.

Giving more attention to this immediate concern, this review is intended to provide a well-organized outline of advantageous and peculiar genetic features for PHA production in *C. necator* H16. Considering the optics, the review is presented as follows: Initially, we will focus on the brief description and distribution of the whole PHA system and its associated constituents reported in the organisms of Genus *Cupriavidus*, as a token of interest to the current subject. Later, the recent findings on proteomics, transcriptomics, and other molecular techniques are briefly explained in relation to the presence of PHAs associated machineries. Subsequently, some potent selected examples are put forward to demonstrate the major groundworks in this field. Finally, a critical discussion on the new knowledge of key genome features and emerging insights into the lifestyle of this bacteria are presented.

Cupriavidus necator H16: the exceptional bioplastics pioneer

The genus *Cupriavidus* comprises more than 15 species (*C. alkaliphilus, C. basilensis, C. campinensis, C. gilardii, C. laharis, C. metallidurans, C. numazuensis, C. oxalaticus, C. pampae, C. pauculus, C. pinatubonensis, C. respiraculi, C. taiwanensis, C. nantongensis, C. necator*) of wide diversity with potential biotechnological applications, especially in biomineralization and bioremediation. In which, the typical PHB producer, *C. necator* H16 is a chemolithotrophic (with CO_2 and H_2 as the sole carbon source and electron donor, respectively), heterotrophic organism capable of growing in CO_2 and H_2 (Pohlmann et al. 2006). It is a non-pathogenic strain compared to other strains belonging to Genus *Cupriavidus*. For instance, *Cupriavidus* species such as *C. pauculus, C. gilardii*, and *C. metallidurans* have been described to cause human infections (Karafin et al. 2010; Langevin et al. 2011). Given the safety concerns and its environmental friendly nature, *C. necator* H16 is suitable for laboratory conditions. On the other hand, PHA producing organisms including *A. vinelandii* and *P. aeruginosa*, synthesize other substances like alginate and rhamnolipid together with PHAs during non-favourable growth conditions provided with excess carbon source (Pham et al. 2004; Galindo et al. 2007). During this process, a considerable portion of 3-HA CoAs are usually directed towards rhamnolipids production, which negatively affected the final concentration of mcl-PHAs (Deziel et al. 2003). Unlike these PHA producers, *C. necator* H16 does not contain any corresponding

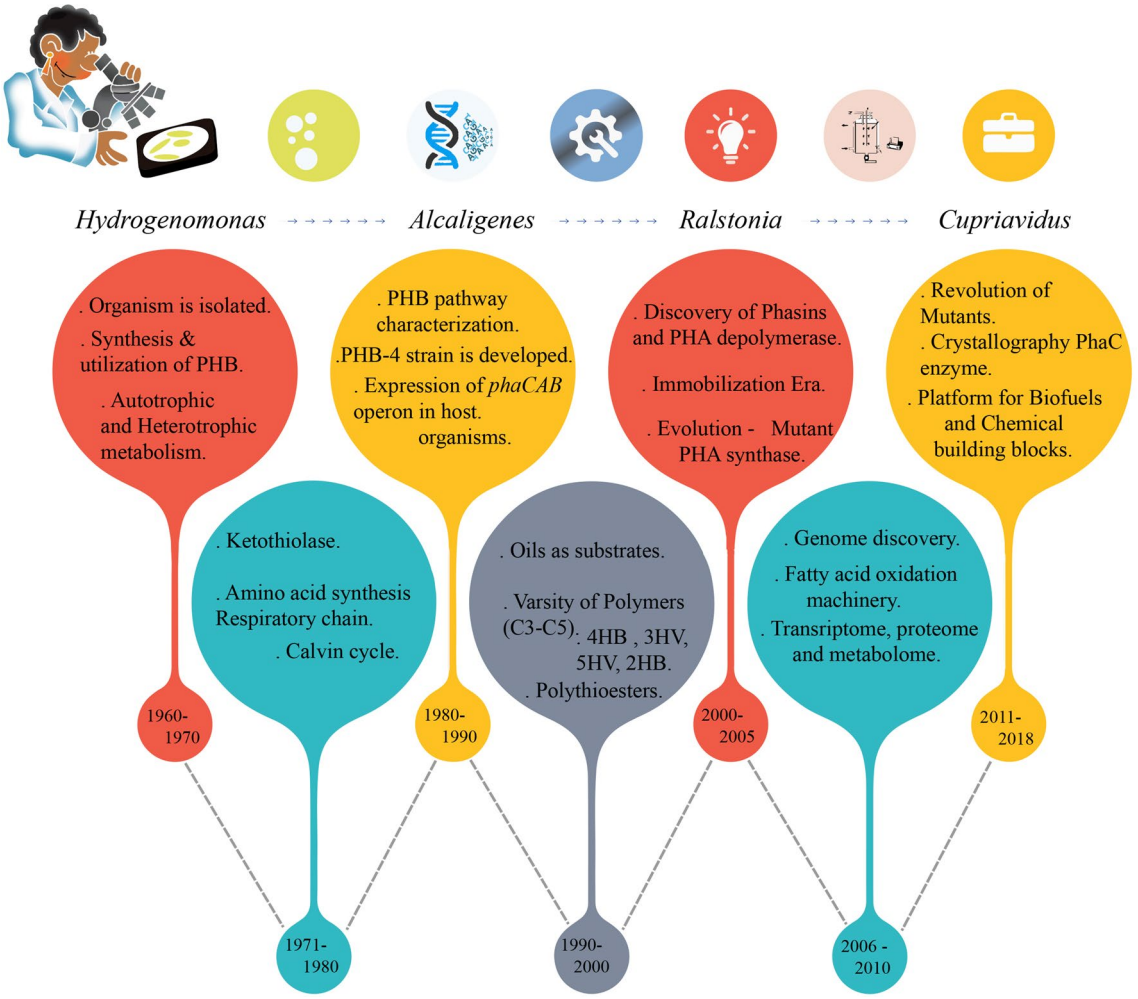

Fig. 1 Timeline representation of *C. necator* H16 organism. A Historical outline representing the significant transitions of *C. necator* H16 organism, happening during 1960 to until now, in relation to the evolution of PHAs

genetic elements for concomitant production of other side substances (Pohlmann et al. 2006). Indeed, the genome features are completely dedicated only to PHAs. Signifying that, the metabolic intermediates as whole can be channelled for PHAs. These notable elements showed certainty that *C. necator* H16 holds upper-hand over other PHAs producers in many ways.

The genome of *C. necator* H16 is encrypted with a high efficiency constitutive $phaC_1AB_1$ operon, encoding enzymes such as PHA synthase (*phaC1*), acetyl-CoA acetyltransferase (*phaA*) and acetoacetyl-CoA reductase (*phaB1*), to synthesize PHB (Peoples and Sinskey 1989). The synthesis occurs through three successive enzymatic reactions as depicted in Fig. 3, which include a condensation of acetyl CoA to acetoacetyl CoA (PhaA) and subsequently reduced by PhaB1 to 3-hydroxybutyrl CoA(3-HB) monomer. Later, PhaC1 proteins polymerize the 3-HB monomers to form PHB granules in numbers of > 15. The genetic information of polymer accumulating ability in Genus *Cupriavidus* is widespread. With respect to this, a bioinformatic analysis, as shown in Fig. 2, identified the conservation of peculiar $phaC_1AB_1$ operon in novel species and they are considered to have potential to accumulate PHAs. Meaning that, the principal source of PHB is from the route governed by the operon in these organisms. As highlighted in Fig. 2, genetics of most *Cupriavidus* species are yet to be characterized in future experimental works.

Profiling *C. necator* H16 at genetic/proteomic level

It was only about a decade ago that the genome was characterized and main thanks to research groups, including Y. Doi, Anthony J. Sinskey, Alexander Steinbuchel, S.Y. Lee, Dieter Jendrossek and T. Fukui, for their extraordinary dedication to unravel the transcriptomic, proteomic and metabolic behaviour of *C. necator* H16 (Brigham et al. 2012;

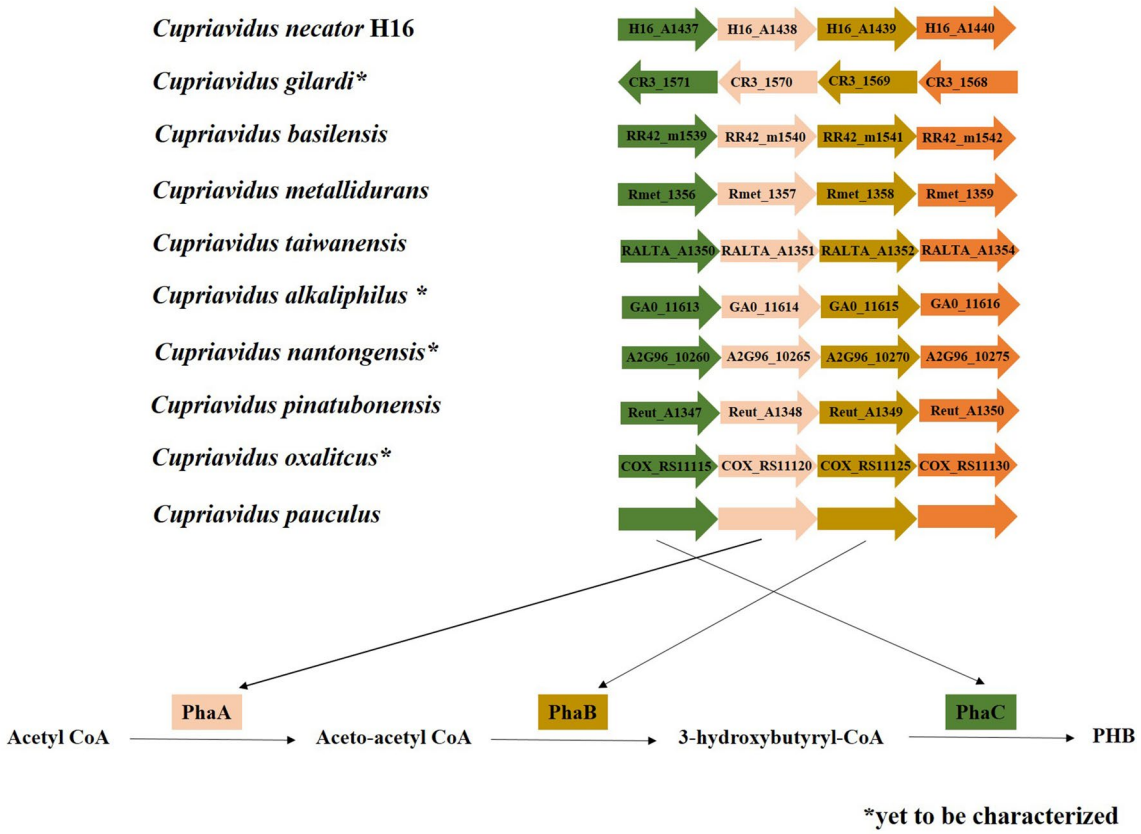

Fig. 2 Distribution and conservation of $phaC_1AB_1$ gene cluster in the Genus *Cupriavidus*. *yet to be characterized

Fukui et al. 2013; Shimizu et al. 2013). The outstanding analyses have identified PHA system specific proteins, fatty acid machinery (oxidation and biosynthesis), stress related factors and non-PHA associated factors (i.e., cell division, transporters). The available pathways of PHA production and their genes distribution in the genome are represented in Fig. 3. Accordingly, the key major elements taking part in the polymer accumulation are presented as: PHB machinery and PHA machinery (fatty acid oxidation and fatty acid synthesis).

PHB machinery: the classical

The abundant enzymes of classic PHB machinery can be classified in four categories depending on the synthesis and on the degradation:

PHA synthase: two PHA synthase genes, *phaC1* and *phaC2*, have been reported in the chromosome 1 of *C. necator* H16 (Pohlmann et al. 2006). The essential PhaC1 enzyme, present in the $phaC_1AB_1$ operon, had been reported to possess a substrate specificity of C3–C5 3-HA monomers in the given PHA accumulative conditions (Haywood et al. 1989). Unlike the most studies reported, few studies have stated the extensive ability of $PhaC1_{R.eu}$ to incorporate mcl-PHA monomers, 3-hydroxyhexanoate (3-HHx, C6), 3-hydroxyoctanoate (3-HO, C8), 3-hydroxydodecanoate (3-HD, C10) monomers (Dennis et al. 1998). The recent crystallographic studies revealed two distinct domains, *N*-terminal domain (RePhaC1ND; M1-R192) and the *C*-terminal domain (RePhaC1CD; G193-A589) (Kim et al. 2017a, b). The indispensable nature of *C*-terminal domain in the activity of synthases is confirmed in both mutational and crystallographic studies (Jahns and Rehm 2009; Kim et al. 2017a). Additionally, single point mutations are adequately performed on $PhaC1_{R.eu}$ to enhance PHAs accumulation and further, to study their affinity towards diverse substrates (Rehm and Steinbüchel 2002; Nomura and Taguchi 2007). $PhaC2_{R.eu}$, the other synthase enzyme, is reported catalytically inactive and unable to accumulate PHB in natural conditions (Pfeiffer and Jendrossek 2012). In late 1980's, PHB-4 strain, a non-PHB accumulating mutant lacking $PhaC1_{R.eu}$ activity, was developed through chemical mutagenesis (Steinbüchel and Schlegel 1989).

PhaA/PhaB homologs: Multiple gene copies encoding β-ketothiolases and acetoacetyl CoA reductases are present in *C. necator* H16 (Brigham et al. 2012). A total of 21 *phaB* and 15 β-ketothiolases homologs are so far identified and only few of them were characterized (Brigham et al. 2012)

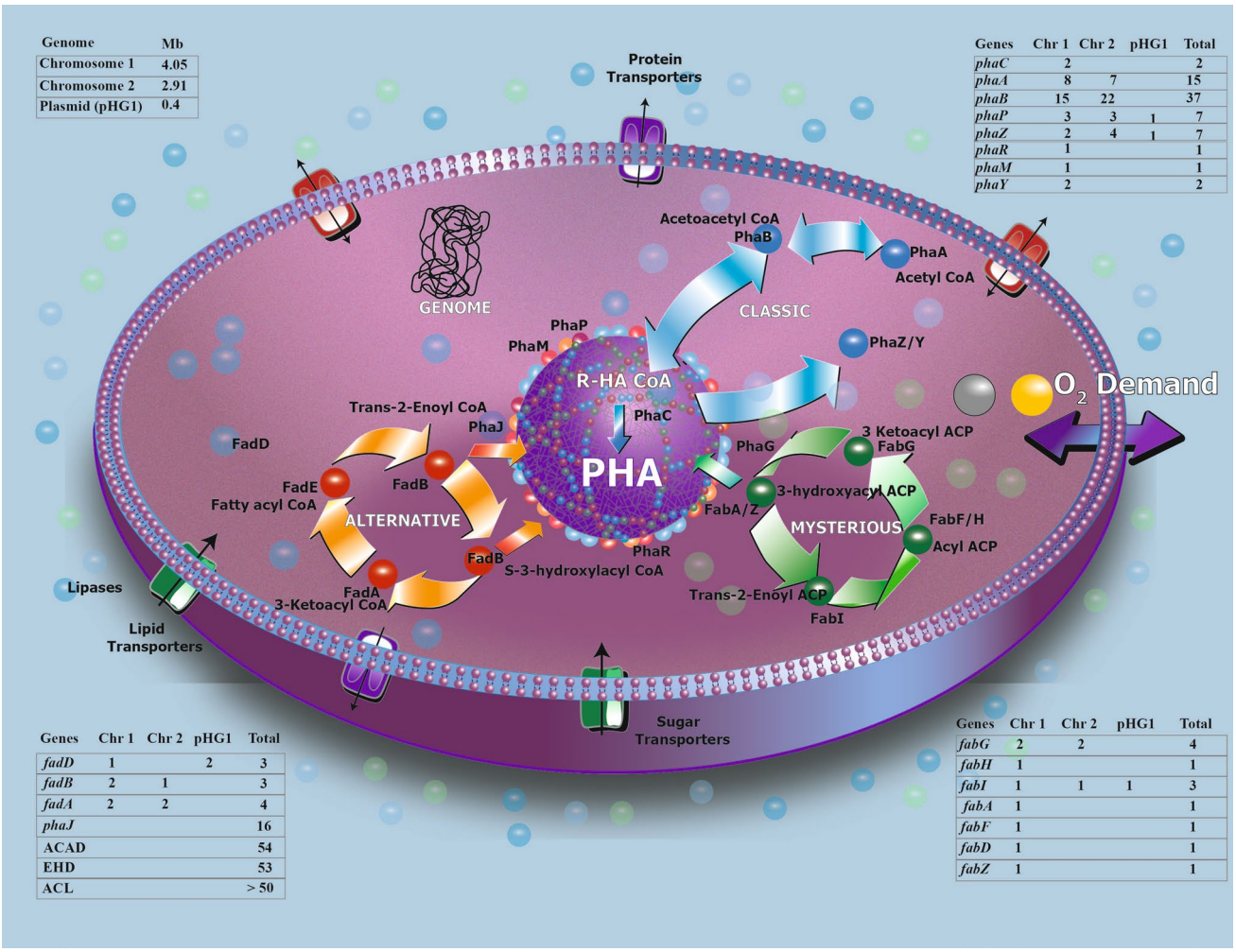

Fig. 3 Various metabolic pathways involved in the PHA synthesis of *C. necator* H16. The R-specific-3-hydroxyacyl CoA monomers derived from various pathways, including fatty acid oxidation and fatty acid synthesis, are polymerized by PhaC synthase enzymes to produce PHAs. The contributing enzymes in the PHA production are shown. The genes distribution of these elements in the genome are tabulated. PhaA—ketothiolase, PhaB—acetoacetylCoA reductase, PhaC—synthase, PhaP—Phasins (PhaP1-7), PhaR—the transcriptional repressor, PhaM—nucleoid separation protein, PhaZ/Y—depolymerases and oligomer hydrolases, FadE—acylCoA dehydrogenase (ACAD), FadB—enoyl CoA hydratase/3-hydroxyacyl dehydrogenase, PhaJ—R specific enoyl CoA dehydratase, FadD—fatty acyl coA ligase (ACL), EHD—enoyl CoA dehydratase, FadA—3-ketoacyl CoA thiolase, FabG—3-oxoacyl-[acyl-carrier-protein] reductase, FabI—Enoyl-[acyl-carrier-protein (ACP)] reductase, FabZ—3-hydroxyacyl-[acyl-carrier-protein] dehydratase, FabA—3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase, FabH/F—3-oxoacyl-[acyl-carrier-protein] synthase

(Fig. 3). The abundances of *phaB* and *phaA* homologs favour PHB production of up to 90% cell dry weight. Among them, PhaB1 and PhaB3 play a major role in supplying 3-HB-CoA monomers (Budde et al. 2010). Also, it is identified that PhaA is the rate limiting enzyme in the PHB pathway involving $phaC_1AB_1$ operon. One of the β-ketothiolase protein, BktB, have diverse substrate specificity and is involved in the formation of 3-ketovaleryl CoA (condensation of propionyl CoA and acetyl CoA) and 3-ketohexanoyl CoA (condensation of butyryl CoA and acetyl CoA) (Slater et al. 1998). The recent findings on PhaB1 led us to understand its ability to reduce 3-ketohexanoyl-CoA in addition to acetoacetyl-CoA, meaning the efficient participation in fatty acid oxidation as monomers' source. In another report, the PhaA and PhaB1 are also been found on the surface of the granules enhancing the channelling of 3-HB monomers to $PhaC1_{R.eu}$ (Uchino et al. 2007).

Phasins: A group of proteins, include Phasins (PhaP) family, PhaR and PhaM, are found on the surface of the granules (Rehm 2006). Over 5% of the total cell protein are reported belonging to Phasins family in PHB accumulating cells. A total of PhaP1–PhaP7 low molecular weight phasins have been identified, in which, PhaP1 is the major surface binding protein (Potter et al. 2004, 2005; Pfeiffer and Jendrossek 2012). Rather than other Phasins, only PhaP1 deletion and overexpression have shown drastic changes

on the PHB granules and accumulation (York et al. 2001). However, pull-down experiments determined that Phasins like PhaP2, PhaP5-7 can have other functions too (Pfeiffer and Jendrossek 2011). PhaR, the transcriptional repressor, controls the expression of both PhaP1 and PhaP3 (York et al. 2002). PhaP3 is found expressed as major protein, only when phaP1 is absent (Potter et al. 2005). PhaM protein facilitates the equal distribution of PHB/PHA granules between cells during cell division by binding of PHB granules to the nucleoid as a scaffold. In addition, PhaM can able to bind with $PhaC_{R.eu}$ and PhaP5 (Bresan and Jendrossek 2017). The specific binding of PhaM with $PhaC1_{R.eu}$ provides enough surface area for interaction with the growing PHA polymer and by which, PhaM activates the synthase enzyme (Pfeiffer and Jendrossek 2014; Kim et al. 2017b). The deletion and overexpression effects of PhaM are most similar to the changes seen with PhaP1 (Bresan and Jendrossek 2017). The reasons for these effects and connections associated with H16ΔPhaM and H16ΔPhaP remains mystery (York et al. 2001; Bresan and Jendrossek 2017).

PHA mobilizing enzymes: the mobilization of intracellular PHAs are carried out by a subset of PHA depolymerases and hydroxybutyrate hydrolase enzymes. The organism possesses five intracellular PHA depolymerases with a DepA domain (PhaZ1–Z5) (Saegusa et al. 2001; York et al. 2003; Abe et al. 2005), two additional depolymerases with an LpqC domain (PhaZ6 and PhaZ7) (Sznajder and Jendrossek 2014) and 2 3HB oligomer hydrolases (PhaY1 and PhaY2) (Kobayashi et al. 2003, 2005). The capacity of intracellular depolymerases to degrade native granules of *C. necator* H16 and artificial amorphous PHB are significantly recognized in various studies. It was proposed that the degradation by depolymerases produce mainly 3-HB oligomers, which are further processed by oligomer hydrolases to monomers (Jendrossek and Handrick 2002). In addition to oligomer hydrolase activity, PhaY1 and PhaY2 are able to degrade artificial granules more similar to PhaZ1 (Kobayashi et al. 2003, 2005). Among different mobilizing enzymes, PhaZ1 (the principal depolymerase), PhaZ2 and PhaZ6 pose high depolymerase activity at both in vitro and in vivo conditions (Abe et al. 2005; Uchino et al. 2008; Sznajder and Jendrossek 2014). So far, PhaZ1, PhaZ6 and PhaY1 (granule and cytoplasm) enzymes are shown effective binding onto the surface of PHB granules.

PHA machinery: the alternative

As depicted in Fig. 3, the genome of *C. necator* H16 contains numerous components involving in the fatty acid oxidation. Extracellular lipase, LipA (H16_A1322) and its associated chaperone (H16_A3742) have demonstrated a significant role in the emulsification of triglycerides to monoacylglycerol, diacylglycerol and free fatty acids present in the oil substrates (Lu et al. 2013). More than 50 copies of fatty acyl CoA ligases are identified, among which, FadD3 seems highly expressed (Pohlmann et al. 2006; Brigham et al. 2010). In addition, *C. necator* H16 poses two operons (*fadEBA*) (H16_A0459-0464; H16A_1526–1531) encoding main fatty acid oxidation components (Fig. 3) (Brigham et al. 2010). They can balance and support the fatty acid metabolism to each other in the absence of either of these operons. Like in model organism *E. coli*, FadE is reported as the rate limiting enzyme in the fatty acid oxidation pathway here too (Heath et al. 2002; Fukui et al. 2013). The genome contains a total of 53 gene copies, encoding enoyl-CoA hydratase enzyme, involved in the generation of (S) and (R)-specific 3-HA CoA intermediates. The fadB1gene, H16_A1526, encoding enoyl-CoA hydratase is also annotated as delta(3)-*cis*-delta(2)-*trans*-enoyl-CoA isomerase, which we believe could participate in the unsaturated fatty acid oxidation pathway. FadB1 (H16_1526) and FadB' (H16_A0461) enzymes are supposed to have higher affinity to 2-enoyl CoAs of scl/mcl and long chain length (Insomphun et al. 2013). Among the 16 copies of PhaJ ((R)- specific enoyl hydratase), $PhaJ_{4aR.eu}$ (H16_A1070) and $PhaJ_{4bR.eu}$ (H16_B0397) displayed major affinity to medium chain length fatty acids (Kawashima et al. 2012). The ability of $PhaJ_{4aR.eu}$ and $PhaJ_{4bR.eu}$ to generate (R)-3-HA CoA monomers holds potential in metabolic engineering strategies. More likely, yet no copies of unsaturated fatty acid oxidation enzymes, like 2, 4-dienoyl CoA reductase (FadH), enoyl CoA isomerase and 3-hydroxyacyl-CoA epimerase required to convert (S)-3-HA CoA to (R)-3-HA CoA, are reported. In turn, multiple copies of fatty acyl-CoA dehydrogenase, 3-ketoacyl CoA thiolase and 3-hydroxyacyl CoA dehydrogenase are also identified in *C. necator* H16 (Pohlmann et al. 2006; Shimizu et al. 2013) (Fig. 3). Surprisingly, it is observed that fatty acid components were active during growth on fructose, implying the role in the turnover of fatty acids (Shimizu et al. 2013). As a result, the medium chain length acyl CoA thioesters were presented in quantifying amounts on fructose. Thus, the abovementioned enzyme machinery promises a possible healthy growth and subsequent PHA production on complex oils and free fatty acids in *C. necator* H16.

PHA machinery: the enigmatic

In *C. necator* H16, the genes *fabHDG-acpP-fabF* (H16_A2569-A2565), *fabZ* (H16_A2044), and *fabI1* (H16_A2410) involved in de novo fatty acid biosynthesis (FAS) were recently identified. In addition, more than 50 copies of acyl CoA synthetase enzymes are recognized. They are highly expressed in the growth phase, but astoundingly many of the genes displayed rather high expression levels in the PHA production phase like fatty acid oxidation components (Fukui et al. 2013; Shimizu et al. 2013). It is in contrary to

the conditions seen in *E. coli*. During unfavourable growth conditions in *E. coli*, the downregulated expression of FAS machinery was highly noted and reported (Heath et al. 2002). Based on the available data, the role of FAS machinery during limitation in *C. necator* H16 remains un-investigated. Also, we predict potential engineering prospects of FAS machinery for 3-HA CoA production, based on the contributions reported earlier in *E. coli* and *P. putida*, using enzymes like PhaG (3-hydroxyacyl-ACP-CoA transacylase) (Li et al. 2007).

Miscellaneous features

The genome also contains a distinct set of metabolic enzymes of high biotechnological interests. A propionate CoA-transferase of *C. necator* H16 with broad substrate specificity catalysing the CoA thioester formation of various carboxylic acids was recently characterized (Lindenkamp et al. 2013). Other enzymes include dihydrolipoamide dehydrogenases (Wübbeler et al. 2010), cysteine dioxygenases (Wenning et al. 2016), aminotransferases (Andreeßen et al. 2017), multifunctional alcohol dehydrogenases and lactate dehydrogenases. The screening of potential short chain dehydrogenases/reductases identified two (S)-enantioselective H16_A1168 and H16_B1297 enzymes showing clear preference towards long-chain and aromatic secondary alcohols, aldehydes and ketones (diaryl diketone benzil) (Magomedova et al. 2016).

Stringent response and PHB accumulation

At laboratory conditions, the cells entering the stationary phase are often encountered with starvation of any kind of nutrient limitation. Such unfavourable conditions alert a stringent response system and provoke a massive sequence of immediate changes for its survival including PHB synthesis in this case. Until this decade, we were very little known about how the events are strictly controlled in *C. necator* H16. Recently, two stringent response proteins, SpoT1, (p)ppGpp synthase/hydrolase and SpoT2, (p)ppGpp synthase, are characterized and shown to regulate the levels of alarmones, such as guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (Juengert et al. 2017). During the nutrient rich period, (p)ppGpp formation is closely regulated by hydrolase activity, wherein the nutrient depletion activates the (p)ppGpp synthase to produce respective alarmones. The alarmones production urges major changes in the transcriptional level of genes to maintain its survival during limitation conditions (Brigham et al. 2012; Shimizu et al. 2013), which are illustrated in Fig. 4. To start, the inhibition of gene transcription by classic transcription sigma factor 70 and the induction of genes by an alternative sigma factor 54 are easily observed. The key physiological changes included downregulation of cell division, transcription and translation machinery, and proteins' secretion, accompanied with the disappearance of flagellin. Genes encoding nitrogen uptake system and carbohydrate metabolism are mostly noted upregulated genes. Metabolically, the high cellular NADH levels decreased citrate synthase activity, thereby arresting Kreb's cycle. The arrest tends to accumulate acetyl CoA molecules, which are further processed by $phaC_1AB_1$ operon enzymes to produce 3-HB CoA and PHB. Subsequently, a norvaline induced stringent response experiment helped us to understand the relation of (p)ppGpp alarmones and the PHB accumulation (Juengert et al. 2017)—the PHB content was detected higher than in the non-treated cells (wild type) which demonstrated a direct relation between them. In addition, excessive levels of PHB were obtained with high alarmones and in absence, the degradation appeared to be faster. These observations showed that the PHB mobilization is very tightly regulated compared to synthesizing it. Supportively, in the double SpoT mutant strain (in the absence of alarmones), the mobilization was unstoppable and occurred in parallel with the synthesis process (Juengert et al. 2017). Several studies demonstrated the significant influence of phasins proteins in the degradation of PHB. It is interesting to conduct a transcriptomic analysis of the depolymerases and phasins proteins in this strain. Hence understood that (p)ppGpp alarmones are important in the PHB accumulation and mobilization. Apart from this, the double SpoT mutants appeared larger and filamentous compared to wild type suggesting an unknown role in the morphology and needs to be investigated.

Past, present and future of *C. necator* H16 in PHAs production

Phase of transition-simpler to complex substrates

At the genetic level, the chromosome 2 encodes genes (H16_B1497-1503; H16_B2504-2507; H16_B1211-1213) for the major metabolizing pathways including Entner–Doudroff pathway, Calvin–Benson–Bassham (CBB) cycle and gluconeogenesis pathway (Pohlmann et al. 2006; Shimizu et al. 2013). It has been identified that the precursors of nucleotides and aromatic amino acids are majorly derived from the CBB pathway. However, enzymes, fructose-1,6-bisphosphate aldolase and 6-phosphogluconate dehydrogenase, corresponding to Emden Meyerhof pathway and pentose phosphate pathway (PEP) are absent. In addition, PEP-dependent phosphotransferase system or a transporter specific for glucose is also absent but glucokinase enzymes to phosphorylate glucose are present (Pohlmann et al. 2006). Hence, its metabolizing ability is restricted to simple substrates like fructose and gluconate. The expression of genes responsible for mannose, glucose metabolism in *C. necator*

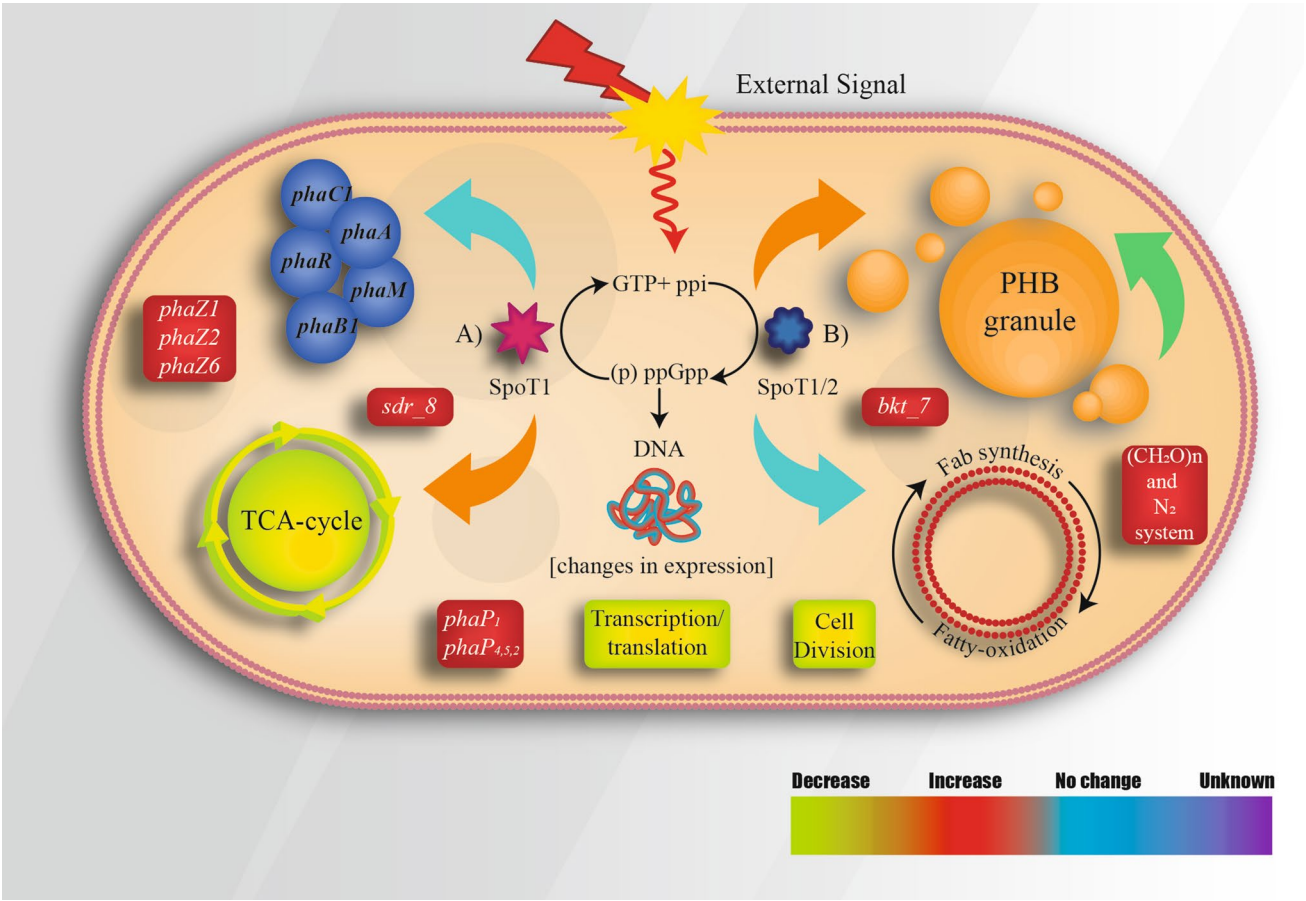

Fig. 4 A schematic representation of stringent response regulation and immediate transcriptional changes during PHA accumulation phase under nitrogen limitation conditions in *C. necator* H16. Two stringent response proteins SpoT1 and SpoT2 have been characterized in the balanced production and recycling of signalling alarmones (p) ppGpp molecules. As shown in (*A*) and (*B*), they switch activities in response to nutrient rich and nutrient depletion conditions. Naturally, the nutrient depletion allows the strains to accumulate PHB with subsequent changes in the expression of genes for its survival. The crucial changes including the upregulation and downregulation of genes expression are highlighted here. (*bkt* ketothiolases; *sdr* short chain dehydrogenases/reductases)

H16 restored the ability to utilize glucose (Sichwart et al. 2011). In a recent review by (Volodina et al. 2016), the versatile engineering of *C. necator* H16 to utilize numerous simple carbon sources such as xylose, arabinose, starch, glycerol, lactose, galactose, starch and lignin derivatives, was exhaustively described. The possibility of metabolizing sucrose by the expression of sucrose hydrolase gene (*cscA*) and sucrose permease gene (*cscB*) from *E. coli* in *C. necator* was demonstrated (Arikawa et al. 2017).

It is highly accepted that *C. necator* H16 accumulate PHB with fructose. In order to generate copolymers, precursors such as sodium propionate, sodium valerate, levulinic acid, 2-hydyxoybutyric acid, g-butyrolactone, 4-hydroxybutyric acid (4HB), 4-chlorobutyric acid and 1,4-butanediol had been extensively utilized. As a result, variety of polymers have been generated; P(3HB-3HV), P(3HB-4HB), P(4HB), P(3HB-3HV-4HB), P(3HB-5HV) (Doi et al. 1990; Reinecke and Steinbuchel 2009). The presence of enzymes, such as dihydrolipoamide dehydrogenases, made possible the use of unusual substrates such as 3,3-thiodipropionic acid 3,3-dithiodipropionic acid, 3-mercaptopropionic, 3,3-dithiopropionic, 3-mercaptobutyric and 3-mercaptovaleric acid to produce mercaptoalkanoates (polythioesters) (Lütke-Eversloh et al. 2001; Lütke-Eversloh and Steinbüchel 2003; Doberstein et al. 2014). Other promising and inexpensive complex carbon substrates are derived from oils, waste streams, crude glycerol, biodiesel and so on. Contrary to sugars, oils are highly rich in fatty acids of carbon length > C10. Attractively, the effective breakdown of fatty acids can supply 3-HA CoA monomers ranging from 3-HB, 3-HHx, 3-HO, 3-HD, 3-HDD and 3-HTD. The generation of multitude of metabolic intermediates can be channelled towards PHAs production (Fig. 3). This knowledge changed the research focus to open new strategies. Such dedicated metabolic engineering strategies on the use of oils and their contributions

on mcl-PHA production in *C. necator* H16 are thoroughly detailed in the following section.

Focus- spotlight on 3-hydroxyhexanoate monomer

The rapid growth of polymer field in the past years and the demand for polymers with better thermal and mechanical properties have provoked researchers to conduct metabolic engineering studies in *C. necator* H16. Though the production of diverse polymers with unusual functional groups is practical, there have been very less information regarding mcl-PHA production, until a sudden breakthrough came-by in late 1990s. A recombinant *C. necator* strain lacking PhaA protein, grown on even chain fatty acids like hexanoic acid (C6) and octanoic acid (C8) as carbon source, accumulated poly(3HB-*co*-3HHx) as storage polymer (Dennis et al. 1998). One of another interesting groundwork came through the consequence of manipulation of PHA synthases and producing PHAs on oil substrates. A recombinant *C. necator* expressing $Pha_{A.ca}$ enzyme, when grown on the free fatty acids such as heptanoic acid (C7), nonanoate (C9) as carbon substrates, produced terpolymer containing monomers of 3HB, 3HV and 3hydroxyheptanoate (Fukui et al. 1997). Followed by this work, Fukui and Doi (1998) verified the ability of growing a PHB-4 strain expressing $PhaC_{A.ca}$ synthase enzyme on diverse oils (corn, olive, palm and oleic). The authors were able to obtain a copolymer of P(3HB-3HHx) with 4–5% of 3HHx fraction. Other investigation shed spotlight on the possibility to accumulate mcl-PHA monomers by arresting fatty acid oxidation using inhibitors like acrylic acid (Green et al. 2002). In the presence of acrylic acid and sodium octanoate, unlike other studies, the native *R. eutropha* strain, without any synthase modifications, accumulated poly(3HP-*co*-3HB-*co*-3HH-*co*-3HO). At the concentration of acrylic acid to 29.3 mM, the authors obtained a tetrapolymer consisting of 6.5 mol% 3HP, 81.7 mol% 3HB, 10.23 mol% 3HH, and 1.6 mol% 3HO. In another study, a recombinant strain PHB-4/pJRDEE32d13, expressing $PhaC_{A.ca}$, had produced a copolymer of 3HB with 5 mol% 3HHx, P(3HB-co-5 mol% 3HHx), from soybean oil as a sole carbon source (Kahar et al. 2004). These works highly recognized that this strain is capable of producing polymers of high quality on oils (rich in fatty acids), when an adequate supply of monomers to a synthase, capable of polymerizing high carbon monomers, is achievable. The vision on this strain had completely changed in identifying new strategies to investigate deeper the mcl-PHA production.

Since then, the advance routes to incorporate 3-HHx monomers, using synthases of *A. caviae* and its variants ($PhaC_{A.ca}$ NSDG;N149S/D171G mutant of PhaCAc) with the capacity of polymerizing C4–C7 substrates (Fukui et al. 1997), had become the centre of attraction because it has been reported that the incorporation of 3-HHx monomers in the growing PHB polymer could guarantee properties as such as commodity plastics. However, to achieve this, researchers were facing serious concerns with the overflow of 3-HB CoA in *C. necator* H16 strain. The stable supply of 3-HB monomers, during limitation conditions by multiple active copies of PhaA and PhaB enzymes, have been a burden in the viewpoint of mcl-PHA production. In particular, PhaB1 and PhaB3, were shown responsible for most of the 3HB-CoA production. Eventually, a series of mutants were created based on PhaA and PhaB homologs for effective reduction of 3-HB CoA supply (Budde et al. 2010; Lindenkamp et al. 2010). Among them, the deletion mutants of H16ΔPhaA(9) and H16ΔPhaB1B2B3 have finally brought a solution to limit the overflow of 3-HB CoA favoring the path for metabolic engineering strategies. Additionally, the principle routes involved in PHB mechanism ($phaC_1AB_1$), forming a critical part, was also engineered by scientists, adding extra support to meet the requirement for mcl-PHA production. The engineering of this operon offers two advantages; (1) The absence of either PhaA, PhaB1 or both limits the production of 3-HB CoA monomers relatively lesser than the wild type strain. (2) The absence of $PhaC1_{R.eu}$ could permit us to engineer the organism with the desired synthase enzymes of higher substrate specificity. Hence, the available mutants formed a host for mcl-PHA production, thereby researchers could engineer with adequate enzymes for channelling the monomers from different routes to the given synthase enzymes.

As a result, many novel mutants, developed in the last decade (Table 1) have been greatly employed and up-do-date information concerning the modification characteristics and the resulting polymers can be found in Table 2. An auxotrophic mutant Re2058/proC, with the deletion of $phaC1_{R.eu}$ and $proC_{R.eu}$ (pyrroline-5-carboxylate reductase) had offered researchers to carry out a positive selection of transformants on proline without the use of antibiotics (Budde et al. 2011). A derivative of the latter strain, Re2058/pCB113, harbouring a plasmid with the *phaC2* of *Rhodococcus aetherivorans, phaJ* of *P. aeruginosa* and *proC* gene for selection had gained attention and evidently been recycled in various investigations on distinct substrates (Table 2). The previously mentioned, ability to grow on oils and its subsequent active fatty acid oxidation pathway enzymes, were highly explored for producing polymers (Table 2). As stated in the literature, most of the works used oils like palm oil, palm kernel oil, soybean oil and canola oil, in which palm kernel oil containing more than 40% of saturated fatty acids was consistently used. Enzymes like PhaJ (*A.ca*, 4a*R.eu* & 4b*R.eu*), PhaB1 and BktB have been expressed to permit the accumulation and channelling of 3-HHx monomers. There have been reports for detectable amount of 3-HO and 3-HDD in the obtained polymers too (Budde et al. 2011).

Table 1 Selected examples of potent *C. necator* H16 mutants for PHAs accumulation and other biotechnological applications

List of potential mutants

Pha synthase; non-PHB accumulating strains

- Re2061 - H16Δ*phaC1AB1*[a]
- Re1034 - H16Δ*phaC1*[a]
- Re2115 - H16Δ*phaC1*[b]
- Re2136 - H16 Δ*phaC1phaB1B2B3*::$phaC_{A.ca}$[b]

Phasins

- Re1052 - H16Δ*phaP1*[c]
- H16Δ*phaP1phaP2phaP3*[d]
- H16Δ*phaP1phaP2phaP3phaP4*[d]
- H16Δ*phaP1phaP2*[d]
- H16Δ*phaM*[e]

Pha Depolymerase

- H16Δ*phaZa1*[f]
- Re1111 - H16Δ*phaZ1Z2Z3*[g]
- Re1112 - H16Δ*phaZ1Z2*[g]
- H16 Δ*phaZ6*[h]
- 05dZ126 - H16Δ*phaZ1Z2Z6*-triple mutant of KNK-005[h]
- 05dZ1246 - H16Δ*phaZ1Z2Z6*-triple mutant of KNK-005[h]
- Re1113 - H16Δ*phaZ2Z3*[g]

Multiple mutants

- Re2133 - H16Δ*phaC1phaB1B2B3*[k]
- Re2058/pCB113 - H16Δ*phaC1proC::proCphaC2*$_{Rsp}$*phaAphaJ*$_{Pae}$[k]
- KNK005 - H16Δ*phaC1*::NSDG mutant of $phaC_{A.ca}$ FA440[h]

PhaA

- H16Δ*phaA_bktB*_H16_A0170[i]
- H16ΔA1713_H16_B1771 _*phaA_bktB*[i]
- H16Δ*phaA_bktB*_H16_A1713_H16_B1771_ H16_B1369_H16_A1528 (_6)[i]
- H16Δ*phaA_bktB*_H16_A1713_H16_B1771_ H16_B1369_H16_A1528_H16_A0170 (_7)[i]
- H16Δ*phaA_bktB*_H16_A1713_H16_B1771_ H16_B1369_H16_A1528_H16_A0170_H16_B0381(_8)[i]

PhaB

- Re2111 - H16Δ*phaB1*[j]
- Re2112 - H16Δ*phaB1B2*[j]
- Re2113 - H16Δ*phaB1B3*[j]
- Re2115 - H16Δ*phaB1B2B3*[j]
- Re2160 - H16Δ*phaB1B2B3proC*[b]

Fatty acid oxidation

- Re2300 - H16Δ(A0459-A0464)[k]
- Re2302 - H16Δ(A1526-A1531)[k]
- Re2303 - Re2300Δ(A1526-A1531)[k]
- Re2312 - H16Δ*fadD3*[k]
- Re2313 - H16ΔA1322[k]

- JM9 - H16Δ(H16_A0459-0464, H16_A1526-1531)Δ*phaC1AB1*[l]

[a]Brigham et al. (2012)
[b]Budde et al. (2011)
[c]Potter et al. (2004)
[d]Potter et al. (2005)
[e]Pfeiffer and Jendrossek (2011)
[f]Saegusa et al. (2001)
[g]York et al. (2003)
[h]Arikawa et al. (2016)
[i]Lindenkamp et al. (2012)
[j]Budde et al. (2010)
[k]Brigham et al. (2010)
[l]Müller et al. (2013)

The increase of 3HHx monomer was found efficient in the co-expression studies of PhaJ than compared to the co-expression of PhaB1, BktB (Table 2). This may be due to the direct participation in the fatty acid oxidation route and broader substrate specificity of employed PhaJ enzymes in engineering approaches. In contrast, the absence of PhaA and PhaB1 operon enzymes in the selected mutant strain (H16Δ$phaC_1AB_1$) have greatly enhanced the production of 3-HHx monomers, without enzyme supplements (i.e. PhaJ) (Mifune et al. 2010). Another approach included the heterologous expression of crotonyl-CoA carboxylase/reductase (ccr), ethylmalonyl CoA decarboxylase (emd) enzymes for efficient channelling of monomers from fatty acid synthesis pathway (Insomphun et al. 2015). In this regard, a series of engineered *C. necator* H16 strains were generated, in which, the enzymes like ccr, emd and $PhaJ_{4aR.eu}$ were expressed alone and together. During nitrogen limitation, the H16Δ*phaB1B3phaC::fabG_bktB_*$phaC_{A.ca}$ NSDG mutant strain expressing the enzymes altogether successfully generated 3-HA monomers from FAS pathway and synthesized P(3HB-3HHx) polymer with higher 37% fraction of 3-HHX,

using fructose as a sole carbon source. Evidently, the overexpression of native PhaJ$_{4aR.eu}$ and PhaJ$_{4bR.eu}$ enzymes have been a great support in the incorporation of mcl-PHA monomers. The additional importance of co-expression of two PHA synthases of distinct substrate specificities in the incorporation of mcl-PHA monomers have been reported (Valdés et al. 2018).

The native *phaP1* and *phaC1* gene promoter have been mostly used as suitable systems for expressing heterologous enzymes in *C. necator* H16 strains (Kawashima et al. 2015). In addition, diverse expression systems have been reported to manipulate the flow of 3-HHx monomers in *C. necator* H16. The expression cassettes based on three different promoter systems (P_{trc}, P_{lacUV5}, P_{trp}) were created to express *phaJ* gene in a mutant strain lacking multiple depolymerase enzymes. The modulation of 3-HHx proportion in the obtained polymers was obvious (Table 3) (Arikawa and Matsumoto 2016). Also, a feeding strategy was conducted using a mixture of 3HV precursors (sodium valerate and sodium propionate) and crude palm kernel oil substrates which resulted in terpolymers P(3HB-3HV-3HHx) (Bhubalan et al. 2008). The factors like feeding time and the concentration of precursors played a critical role in varying the 3HHx monomer composition (Tables 2, 3). The polymer containing 3HHx monomers in various proportions are highly noted and the varying thermal polymer properties are presented in Fig. 5. As expected, the incorporation of 3-HHx monomers have significantly improved thermal properties compared to traditional PHB and copolymer P(3HB-3HV). The gradual changes in the thermal properties with respect to the varying 3-HHx proportion can also easily been seen in the Table 3. It has been understood and also supported in various studies that even the presence of smaller fraction of mcl-monomers have potential impact on the flexibility and thermal properties of the polymers containing higher 3-HB and 3-HV fraction (Table 3). To develop novel fed strategies, one should keenly consider the optimal concentrations of mcl-PHA monomers available to the synthase. Regarding this, the strategies to express the exogenous enzymes i.e., PhaJ with multiple promoter systems could be of helpful to regulate the composition (Wang et al. 2013). The recent metabolic profiling studies demonstrated the higher supply of 3-HHx monomers in the earlier stages of *C. necator* H16 which is valuable to design both expression and feeding strategies (Fukui et al. 2013; Shimizu et al. 2013). Also, the contribution of two 3-keto-thiolases and two acetoacetyl-CoA reductases with the substrate specificities towards C4–C6 substrates, to provide the 3-HHx monomer from fatty acid oxidation could be considered for future approaches. From our point of view, the effective engineering of fatty acid oxidation and FAS pathways have been successful in the production of polymers with 3-HHx mcl-monomer, which is one of the major advancement that *C. necator* H16 have ever seen.

Besides, the engineering of these mutants has been recognized in other studies too. The available H16Δ*phaA* mutants were widely used in the production of polymers with varying 3-mercaptopropionic acid and 3-HV units (Lindenkamp et al. 2010, 2012). Captivating the nature of H16Δ*phaC$_1$AB$_1$* mutant strain, enzymes like mutational PhaC$_{R.eu}$ (S506G/A510K) or the *Pseudomonas* sp. MBEL 6-19 PhaC1437 was coexpressed with pct540 (propionyl CoA transferase) of *Clostridium* to incorporate 2-HB in the polymer (Park et al. 2013). The growth of engineered strain on lactate or 2-HB as substrates produced polymers of monomers ranging from 3HB, 3HV, 2HB and lactate. The coexpression led to a higher incorporation of more than 60% fractions of 2-HB in the polymer.

"Phasins - the hidden figures" behind controlling monomer composition of PHAs

Furthermore, to enhance mcl-PHA polymer production, promising engineering of Phasins come into play together. Previously, it has been demonstrated that the overexpression of PhaP1$_{R.eu}$ promoted PHB production as well as decreased granules sizes and increased number (York et al. 2002). Recent reviews have described the use of Phasins as immobilization anchor for proteins of biotechnological interest (Wei et al. 2011; Jahns and Rehm 2012). Other studies also investigated the influencing role of PhaP1$_{R.eu}$ in the mobilization of PHAs and their relation to depolymerase enzymes (Kuchta et al. 2007). Other functions as chaperones and surfactants were recognized in the recent years too (Wei et al. 2011; Mezzina et al. 2015). In the continual search of other activities of Phasins, a recent study demonstrated that the they can act as physiological activators of PHA synthases (Ushimaru et al. 2014). At in vitro conditions, PhaP1$_{R.eu}$ lacked the ability to activate the PhaC$_{A.ca}$ synthase protein, whereas PhaP$_{A.ca}$ activated the later protein reducing lag phase of polymerization. This activating effect can signify a new role of PhaPs in the polymerization reaction and altering monomers affinity by synthases. These promising results provided new opportunities in the viewpoint of metabolic engineering and motivated to do further modifications in *C. necator H16*.

Kawashima et al. (2015) replaced PhaP1$_{R.eu}$ with PhaP1$_{A.ca}$ in modified strains where in one strain, the Pha$_{A.ca}$ NSDG synthase enzyme was expressed alone and in another, expressed together with PhaJ$_{A.ca}$. Authors reported that the increase of 3HHx fraction was slightly larger than that obtained by the strain harbouring *phaJAc* within *pha* operon and the strain harbouring PhaP$_{R.eu}$PhaJ$_{A.ca}$. This means that the replacement of phasins have strongly increased the 3-HHx content in the polymer without the supplement of

Table 2 Comparison of PHA accumulation by *C. necator* H16 and its modified strains

Strain	Modifications	Fermentation	Substrate	Cell content (g/l)	PHA content (%)	3HB (mol%)	3HV (mol%)	3HH (mol%)	3HO (mol%)	References
Re2058/pCB113[a,b] (H16Δ*phaC1proC*)	$PhaC2_{R.ae}$ $PhaJ_{P.ae}$, ProC	Fermenter	4% Palm oil	3.6	71	88		12	TA	Budde et al. (2011)
Re2160/pCB113[a,b] (H16Δ*phaC1B1B2B3proC*)	$PhaC2_{R.ae}$, $PhaJ_{P.ae}$, ProC	Fermenter	4.5% Palm oil	2.74	66	76		24	TA	
Re2058/pCB113		Fermenter	5 g Olein	5.13	67	73		27		Murugan et al. (2017)
			5 g Fructose	2.32	11	100		–		
Re2058/pCB113		Shake flask	CPKO	5.82	73	58		42		Murugan et al. 2016
			2% oil palm tree trunk sap sugars	6.99	63	73		27		
Re2160/pCB113		Shake flask	2.5 g/l CPKO	2.7	45	32		68		Wong et al. (2012)
			Cocunut oil	2.6	48	20		70		
Re2135 (H16Δ*phaCB1B2B3*)	$PhaC2_{R.ae}$	Shake flask	Coffee waste oil	0.93	69	78		22		Bhatia et al. (2018)
Re2135 (H16Δ*phaCB1B2B3*)	$PhaC2_{R.ae}$	Shake flask	Butyrate	0.1	4	71	16.5	12.5		Jeon et al. (2014)
Re2058/pCB113[b]		Fermenter	Palm oil	98	70	76		24		Riedel et al. (2012)
Wild type H16		Shake flask	Jatropha Oil/ sodium valerate	6.7	76	58	42			Ng et al. (2011)
PHB-4/pBBREE32d13	$PhaC_{A.ca}$	Shake flask	Jatropha oil	8	84	97		3		
			CPKO	4.3	87	95		5		
PHB-4/pBBREE32d13	$PhaC_{Chr}$	Shake flask	Sodium valerate/ CPKO	4.3	47	73	22	5		Bhubalan et al. (2008)
			Sodium propionate/CPKO	3	30	86	12	2		
C. necator H16		Shake flask	5 g/l Palm acid oil		79	100				Kek et al. (2008)
			5 g/l Palm kernel acid oil							
PHB-4/pBBREE32d13[b]	$PhaC_{A.ca}$	Fermenter	Soybean oil	138	74	95		5		Kahar et al. (2004)
C. necator H16		Shake flask	Waste frying oil			100				Verlinden et al. (2011)
C. necator H16[c]		5 l Fermenter (3 stage)	Canola oil	92	48	99.81	0.06	0.09	0.04	Rathinasabapathy et al. (2014)
C. necator H16[c]	$PhaC2_{P.pu}$ CA-3	5 l Fermenter (3 stage)	Canola oil	6.2	49	96.5	0.8	2.7		Valdés et al. (2018)
PHB-4	$PhaC2_{P.pu}$ CA-3			3.7	96	94	1	4	1	
C. necator H16[c]		Fermenter	Canola oil	20	92	95		5 (UD)		López Cuellar et al. (2011)
H16Δ*phaC1*	$PhaC_{A.ca}$	Shake flask	0.5% Soybean oil	2.6	72	98		2.00		Mifune et al. (2008)
			0.5% Octanoate	4.1	87	89.2		10.8		
PHB-4/pBBREE32d13	$PhaC_{A.ca}$	Shake flask	0.5% Soybean oil	2.5	77	97.5		2.5		
			0.5% Octanoate	4.4	84	87		13		

Table 2 (continued)

Strain	Modifications	Fermentation	Substrate	Cell content (g/l)	PHA content (%)	3HB (mol%)	3HV (mol%)	3HH (mol%)	3HO (mol%)	References
H16Δ*fadB1*	$PhaC_{A.ca}$ NSDG	Fermenter	Soybean oil	6.6	86	97.4		2.6		Insomphun et al. (2013)
H16Δ*fadB2*				6.6	88	98.6		1.4		
H16Δ*fadB1B2*				6.4	88	98.1		1.1		
H16Δ*phaC1fadB1*	$PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$			4.6	90	89.2		10.8		
H16Δ*phaC1AfadB1*	$PhaC_{A.ca}$ NSDG, $PhaJ_{4aR.eu}$			5.1	84	90.7		9.3		
	$PhaC_{A.ca}$ NSDG, $PhaJ_{4bR.eu}$			4.7	82	88.3		11.7		
H16Δ*phaC1AB1*[a]	$PhaC_{A.ca}$ NSDG	Shake flask	1% Soybean oil	0.9	26	74.3		25.7		Mifune et al. (2010)
H16Δ*phaC1A*	$PhaC_{A.ca}$ NSDG, BktB			5.1	82	97.4		2.6		
H16Δ*phaC1*	$PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$			4.8	79	90.1		9.9		
H16Δ*phaC1*[b]	$PhaC_{A.ca}$ NSDG	5 l Fermenter; 3L MB medium	PKO	171	78.1	92.8		7.2		Sato et al. (2015)
H16Δ*phaC1A*	$PhaC_{A.ca}$ NSDG			165	73.3	92.3		7.7		
H16Δ*phaC1*	$PhaC_{A.ca}$ NSDG, BktB			170	77.7	92.6		7.5		
	$PhaC_{A.ca}$ NSDG, BktB		PKO and Butyrate	170	76.5	87		13		
H16Δ*phaC1Z1Z2Z6*	$PhaC_{A.ca}$ NSDG			19.1	84	97.2		2.8		Arikawa et al. (2016)
	PtrcRBS-$PhaJ4b_{R.eu}$			18.2	84	89.3		10.7		
H16Δ*phaC1*	$PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$	Shake flask	1% Soybean oil	5.7	84	89.5		10.5		Kawashima et al. (2015)
H16Δ*phaC1phaP1*	$PhaP_{A.ca}$ $PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$			6.3	79	82.8		17.2		
MF02 (H16Δ*phaC1*)	$PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$			6.8	88	96.4		4.6		
MF02-PAc	$PhaP_{A.ca}$ $PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$			7.2	90	94.6		6.4		

Table 2 (continued)

Strain	Modifications	Fermentation	Substrate	Cell content (g/l)	PHA content (%)	3HB (mol%)	3HV (mol%)	3HH (mol%)	3HO (mol%)	References
H16Δ*phaC1B1B3*[a]	$PhaC_{A.ca}$ NSDG, $FabG_{Ps}$ BktB, Ccr_{Me}	Shake flask	0.5% Fructose	0.96	4.4	81.9		18.1		Insomphun et al. (2015)
	$PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$, BktB, Ccr_{Me}			0.96	11.4	82.9		17.1		
	$PhaC_{A.ca}$ NSDG, BktB			0.98	4.4	80.9		19.1		
	$PhaC_{A.ca}$ NSDG, $PhaJ_{A.ca}$, Ccr_{Me}, emd			1.42	41	62.3		37.7		

PKO palm kernel oil, *CPKO* crude palm kernel oil, *R.ae Rhodococcus aetherivorans, A.ca Aeromonas caviae*, P.pu *Pseudomonas putida, ccr* crotonylCoA carboxylase/reductase, *emd* ethylmalonyl CoA decarboxylase, *proC* pyrroline-5-carboxylate reductase, *BktB* ketothiolase, *PhaJ* enoyl CoA hydratase, *FabG* 3-oxoacyl-[acyl-carrier-protein] reductase, *PhaP* Phasin, *PhaZ* depolymerase, *TA* trace amounts, *UD* undetermined

[a]Indicates that the knowledge of metabolic engineering can be abducted to enhance PHAs in future studies

[b]Two-stage fermentation

[c]Three-stage fermentation

any other metabolic enzymes associated for 3-HHx supply (Kawashima et al. 2015) (Table 3). However, the phasin proteins replacement reduced the molecular weight of the polymer. We hypothesize that the noted reduction in the molecular weight might be due to the absence of native $PhaP1_{R.eu}$ expecting a higher degradation rate, which must be considered for future ideas (Kuchta et al. 2007). Additionally, the production of alternate monomers other than 3-HHx in bulk quantities has not yet been reported. The successful attempt ultimately comprehend that in different host organisms, the addition of respective Phasin partners (i.e. $PhaP_{A.ca}$) to synthase (i.e. $PhaC_{A.ca}$) enzymes has the potential to improve the proportion of mcl-PHA monomers in the resulting polymer (Kawashima et al. 2015). Despite the $PhaP1_{R.eu}$, there is a compelling evidence suggesting the possible role of PhaM as physiological activator of $PhaC_{R.eu}$ synthases (Pfeiffer and Jendrossek 2014; Kim et al. 2017b) and must be accounted in near future. These studies also aided a certainty that: in addition to the PHA synthase polymerizing nature, several factors like Phasins could be able to control the desired polymer composition.

Influence of large scale fermentation

The practice of flask and scale-up bioreactor fermentations are familiar and widespread in this polymer field. We comprehend from literature that the key knowledge, regarding the effects of genetic modifications on the growth and PHAs accumulation ability, are often obtained from the traditional flasks. Once accomplished in flasks, fermentations are carried out in bioreactors to enhance the PHA productivity by achieving a high cell density (HCD). The following factors associated with bioreactors merit flasks in the perspective of HCD: (1) The temperature, pH, dissolved oxygen conditions are controllable, and maintenance of mass transfer of the gases is possible. (2) The required higher oxygen demand for cell growth and subsequent PHA production on oil substrates could easily be met. (3) The feeding strategies involving co-mixtures of substrates can be well-controlled in fermenter. (4) Foaming can be controlled by adding antifoaming agents and by adequate baffles. Considering the given advantages, two-stage fermentation (Kahar et al. 2004; Budde et al. 2011) and three-stage fermentation strategies (Ryu et al. 1997; López-Cuellar et al. 2011; Rathinasabapathy et al. 2014) have been proposed already to achieve HCD cultures in bioreactors and are in practice. Some of the key examples are presented in Table 2. The fermentation strategies have greatly enhanced the mcl-PHA incorporation in either with or without any external genetic modifications (Table 2). It is believed that the HCD cultures would have substantial number of secreted lipases in the extracellular medium favouring the efficient emulsification process and thereby, oil consumption and PHA productivity. The notable

Table 3 Comparison of thermal properties with respect to the varying 3-HA composition and proportion

Polymer	T_{ga} (°C)	T_{ma} (°C)	ΔH_{ma} (Jg^{-1})
P(3HB)[a]	4	180	60–80
P(3HB-co-2 mol% 3HV)[b]	− 0.7	168	65.2
P(3HB-co-3 mol% 3HV)[b]	− 0.9	168	59.5
P(3HB-co-4 mol% 3HV)[b]	− 0.5	170	59.1
P(3HB-co-5 mol% 3HV)[b]	− 0.2	169	56.8
P(3HB-co-7 mol% 3HV)[b]	− 0.8	166	64.7
P(3HB-co-7 mol% 3HV)[b]	− 0.2	167	54.5
P(3HB-co-8 mol% 3HV)[b]	− 1.6	169	41.5
P(3HB-co-8 mol% 3HV)[b]	− 1.2	169	49
P(3HB-co-1.6% 3HHx)[c]	8	162	65
P(3HB-co-2 mol% 3HHx)[d]	1.8	146, 158	50.2
P(3HB-co-2.7 mol% 3HHx)[e]	− 2.3	150.8	72.9
P(3HB-co-4% 3HHx)[f]	− 1	164	54
P(3HB-co-5.2 mol% 3HHx)[e]	− 2.9	142	70.8
P(3HB-co-5.9 mol% 3HHx)[e]	− 2.4	138.9	69.5
P(3HB-co-7.1 mol% 3HHx)[e]	− 2.1	133.5	66.3
P(3HB-co-7.9 mol% 3HHx)[e]	− 2.2	131.1	67.5
P(3HB-co-27% 3HHx)[f]	− 1	120	18
P(3HB-co-60% 3HHx)[g]	− 11	ND	ND
P(3HB-co-70% 3HHx)[g]	− 12	ND	ND
P(3HB-co-3 mol% 3HV-co-3 mol% 3HHx)[h]	− 3.8	129, 144	60
P(3HB-co-15 mol% 3HV-co-3 mol% 3HHx)[h]	− 2.3	147	41
P(3HB-co-24 mol% 3HV-co-7 mol% 3HHx)[h]	− 0.8	129, 139	28
P(3HB-co-32 mol% 3HV-co-2 mol% 3HHx)[h]	− 4.7	91, 148	66
P(3HB-co-85 mol% 3HV-co-1 mol% 3HHx)[h]	− 16.1	89	48
PHB-co-PHV-co-PHHx-co-PHO[i]	− 14	155–173	30

ND not determined

[a]Doi et al. (1990)

[b]Lee et al. (2008)

[c]Ng et al. (2011)

[d]Chuah et al. (2013)

[e]Arikawa et al. (2016)

[f]Murugan et al. (2017)

[g]Wong et al. (2012)

[h]Bhubalan et al. (2010)

[i]Valdés et al. (2018)

differences in the production of polymers from flasks and fermenters can be seen in Table 2. In addition, the challenges in the downstream processing can partly be reduced by higher biomass production. The HCD favours the production of sufficient polymeric material that would be helpful to study mechanical properties. From our experience, even the presence of monomers in the minor quantities can be detected, when the polymer is extracted at large scale.

However, plasmids stability is the serious concern on industrial scale fermentations. Reports stating the loss of plasmids after certain period of fermentations are available. Taking this into account, few studies have already intended to bring novel plasmids system for selection without antibiotics. The previously mentioned auxotrophic mutant using proline system protein (Budde et al. 2011) and the partition locus (comprising *parA*28 and *parB*28 and the *parS*28 region) from *C. metallidurans* (Sato et al. 2013) are suitable examples for plasmid maintenance. Convincingly with these understandings, the progress of scaling up fermentation is evident and healthy for this field. Hence, the added knowledge on the good engineering of strains, substrate selection, and the cultivation strategy that one handles are more critical to generate high quality polymers.

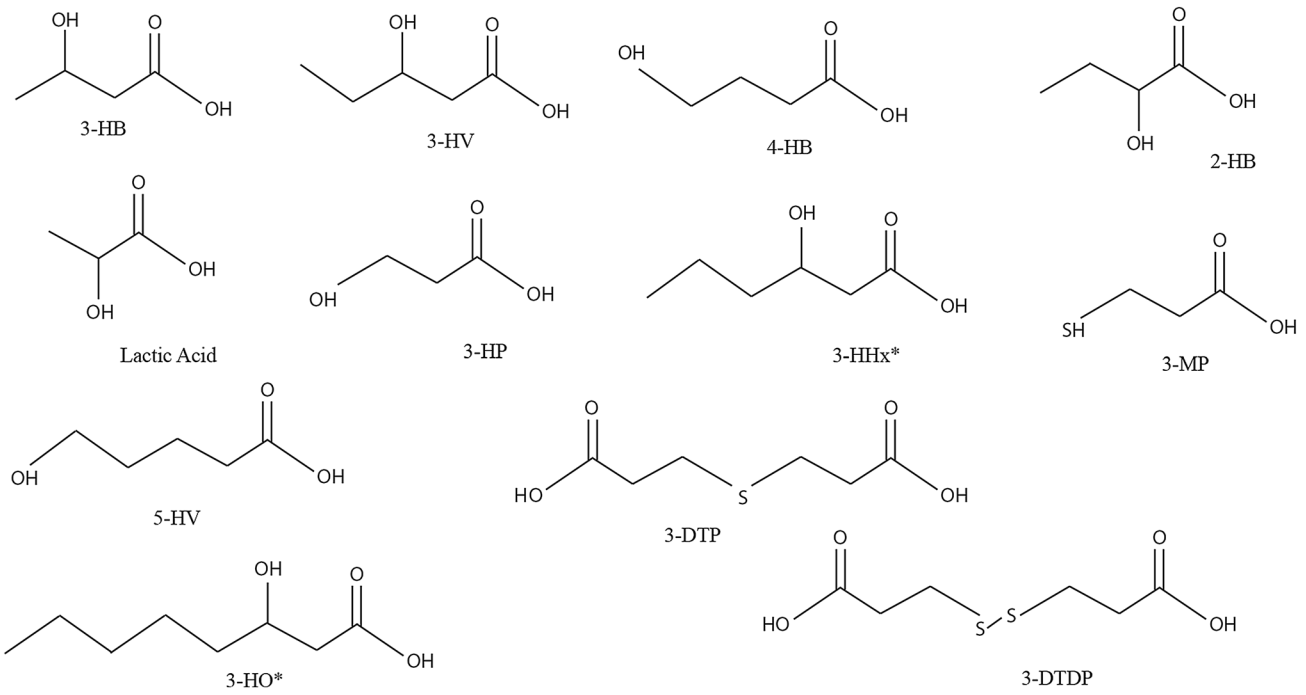

Fig. 5 Commonly synthesized scl-PHA and mcl-PHA monomers in the *C. necator* H16. *Indicates mcl-PHA monomers

PhaZ – "last but not least"

The synthesis of high-molecular-weight PHA polymers are largely preferred, as they possess improved mechanical properties, contributing to high modulus and high strength of the drawn materials. So far, the use of substrates like glycerol and polyethylene glycol in the culture medium have significantly reduced molecular weight than the polymer produced in glucose (Shi et al. 1996; Madden et al. 1999). This effect has been attributed to chain termination caused by glycerol (Madden et al. 1999). Other considerable key factors to control polymer properties are type of nutrient limitation, the concentration of PHA synthases, the catalytic activity of PHA synthase(s) and the simultaneous degradation of PHA during biosynthesis (Asenjo et al. 1995; Sim et al. 1997). Since in *C. necator* H16, PhaZ proteins are known to mobilize intracellular polymer in parallel to synthesis, the question arises about their role in the regulation of molecular weight of the polymer. Until now, we have known that mobilizing enzymes (PhaZ 1–7 and PhaY 1&2) are expressed higher in the PHA production phase than in the utilization phase (Brigham et al. 2012) (Fig. 4). Especially, the distinctive expression states of PhaZ6 proteins on fructose (low) and oil (high) substrates are noted. In addition, the degradation rate was observed very less in the deletion mutant of H16Δ*phaZ1* (Abe et al. 2005). The single deletion mutants of PhaZ (Z6, Z7) and PhaY (1&2) proteins don't shown any adverse effects on the growth but slightly reduced the PHA content (Kobayashi et al. 2005; Sznajder and Jendrossek 2014).

Taking this into account, (Arikawa et al. 2016) performed a study to investigate the relationship between intracellular PHB mobilization enzymes and molecular weight of PHAs accumulating in *C. necator* H16. In this regard, a series of *phaZ* mutants of KNK00505 (See Table 1) and *C. necator* H16 were cultivated. In the H16Δ*phaZ1* mutant, the PHA content was relatively higher as seen in other studies than wild type but there was no change in the molecular weight. Surprisingly, the strain H16Δ*phaC1* expressing $PhaC_{A.ca}$NSDG synthase have significant higher PHA content and molecular weight than any single deletion mutant strains of PhaZ(1–7). Compared to other studies and developed mutants, only H16Δ*phaZ6* mutant and a multiple mutant, KNK00505Δ*phaZ1Z2Z6*, produced ultra-high-molecular-weight polymer P(3HB-3HHx) from fructose and palm kernel oil, which was approximately twofold or more than the parent strains did. It is important to note that the differential expression profile of PhaZ6 on different substrates have played an uncertain role in the regulation of molecular weight. The barely degrading nature of the polymer enzymatically intracellularly in these strains could have aided the continual accumulation of PHAs resulting in high molecular weight. More likely, authors also have suggested that degradation would have happened only on the surface of granules. They also confirmed that PHA synthase activity ($PhaC_{A.ca}$NSDG) do not alter the molecular weight of PHAs

in the PhaZ deletion mutant strains. Overall, the current finding opened another feasible strategy, becoming a proof-of-concept, for efficient modulation of polymer molecular weight by simple arresting of enzymatic hydrolysis through the deletion of *phaZ* genes encoding depolymerase enzymes on oil substrates (Arikawa et al. 2016).

Recharging mutant strains: the appreciable act

Since the discovery of the mutants, the fellow-researchers conceived the idea of gaining profit from the acquired knowledge in applications besides PHA production. As an outcome, recent years have witnessed novel drop-in fuels production and chemicals synthesis pathways employing genetic engineering approaches, where the implication of the nature of mutants played a vital role. Especially, the natural ability of various mutants including H16Δ *phaC*$_1$*AB*$_1$ and H16Δ*phaC1* to accumulate 3-HB CoA monomers during nutrient limitation were extensively utilized rather than the wild type strain. The first examination was the production of 2-methylcitric acid utilizing the mutants of *R. eutropha* (Ewering et al. 2006). In response to growing concerns from the scientific community regarding drop-in fuels, various studies have described strategies to produce isobutanol. An engineered strain, named *R. eutropha* LH74D, was created, by introducing genes responsible for isobutanol and 3-methyl-1-butanol (3 MB) production, in *C. necator* H16Δ*phaC*$_1$*AB*$_1$ strain (Li et al. 2012). Later, the strain, grown in a pH-coupled formic acid feeding fermenter, produced fuels with the final titer of over 1.4 g/l (~ 846 mg/l isobutanol and ~ 570 mg/l 3 MB). In other approach, the same strain produced over 140 mg/l biofuels in an integrated electromicrobial reactor (powered with the electrochemical CO_2 reduction on the cathode to produce formate) with electricity.

To improve the biosynthesis of isobutanol and 3 MB, genes encoding three potential carbon sinks (*ilvE, bkdAB*, and *aceE*) were further eliminated in the mutant strains of *C. necator* H16 (Lu et al. 2012). Then, a plasmid-borne overexpression system comprising, native isobutyraldehyde dehydrogenase, branched-chain amino acid biosynthesis pathway genes (*ilvBHCD* and *kivD*) and the heterologous ketoisovalerate decarboxylase genes, was constructed and fed into mutants. Over the duration of 50 days in semi-continous culture, authors achieved a successful production of 14 g/l of branched chain alcohols. Another study on the same H16Δ *phaC*$_1$*AB*$_1$ strain, the heterologous expression of alcohol dehydrogenases from *E. coli* using acetate as sole carbon source produced ethanol (Lee et al. 2016). Focusing on the production of isopropanol, a strain named Re2133/pEG7c was developed, overexpressing native *C. necator* genes encoding a β-ketothiolase, a CoA-transferase, and codon-optimized *Clostridium* genes encoding an acetoacetate carboxylase and an alcohol dehydrogenase (Grousseau et al. 2014). Using fructose as a sole carbon source, the modified strain was able to generate up to 3.44 g l^{-1} isopropanol in batch culture. In a subsequent attempt, the over-expression of native chaperone (GroESL) together with the isopropanol pathway genes enabled the yield of 9–18% on fructose (Marc et al. 2017). Comparatively, a final isopropanol concentration of 9.8 g l^{-1} was achieved in fed-batch culture. This study is a proof-of-concept and easily adaptable to other drop-in fuels production that the overexpression of the native chaperones improved the tolerance of an organism, beyond the higher titers of given end-product i.e., isopropanol and thereby, the cell viability as well.

In another study, the overflow of 3-HB CoA in the strains was directed to 2-hydroxyisobutyrate (2HIB). In this regard, the ability of enzymes, cobalmin-dependent mutase (Hoefel et al. 2010) and vitamin B12-dependent 2-hydroxyisobutyryl-coenzmye A (2-HIB-CoA) mutases (Przybylski et al. 2013), catalyzing the isomerization of 3-HB CoA to 2HIB CoA were captivated. Various strains, including wild type, PHB-4 strains, H16Δ*phaC1* mutant strain were tested using fructose and butyrate as substrates (Przybylski et al. 2013). Authors reported the 2HIB production was higher and efficient in PHB-4 strain than in any other strains. This can be attributed to presence of reduced activity of PhaB1 and continual accumulation of acetoacetyl CoA in PHB-4 strain. Under chemolithotrophic conditions in a modified *C. necator* H16 strain, the coupled reaction of enzymes, native NAD-reducing hydrogenase and heterologously expressed alcohol dehydrogenase from *Kluyveromyces lactis*, produced 1,2 propanediol from hydroxyacetone (Oda et al. 2013). Adding potential to the H16Δ*phaC*$_1$*AB*$_1$ strain, a quite variety of alkanes were generated by a successful transfer and expression of alkane synthesis pathway genes coding an acyl-ACP (acyl carrier protein) reductase and an aldehyde deformylating oxygenase from *Synechococcus elongatus* (Crépin et al. 2016). The expression of non-native genes on fructose under nitrogen limitation produced alkanes and alkenes consisting of pentadecane, heptadecene, heptadecane and hexadecanal.

Alternatively, Müller et al. (2013) highlighted the importance of engineering fatty acid pathways in the production of methylketone. To achieve methyl ketone production in a fatty acid operon deleted strain, TesA (Thioesterase) was overexpressed together with the acyl coenzyme A oxidase from Micrococcus luteus, FadB and FadM (Long-chain acyl-CoA thioesterase) from *E. coli*. The strain produced 50–65 mg/liter methyl ketones under heterotrophic growth conditions and 50–180 mg/liter under chemolithoautotrophic growth conditions (Müller et al. 2013). Finally, there is evidence for the efficient generation of medium chain length fatty acids from a H16Δ*phaC*$_1$*AB*$_1$ strain lacking highly upregulated fatty acyl coA ligase *fadD3* (Chen et al. 2015). The coexpression of *UcFatB2*, a medium-chain-length-specific

acyl-ACP thioesterase, in the strain H16Δ*phaC*$_1$*AB*$_1$*fad3*, produced 62 mg/l of medium chain length fatty acids.

Hence, these bioprospecting strategies did a fair trade with the mutant strains of *C. necator* H16 to lay a platform for a wide range of value added products, just by making a proper channelling route to the heterologous expressed enzymes. It is proven that the vital resources of *C. necator* H16 is not only limited to PHA synthesis and perhaps can be beneficial and explored in various other biotechnological applications. The adequate knowledge of fatty acid oxidation and FAS machinery could be directed towards the production of other bulk materials including 3-hydoxycarboxylic acids and fatty alcohols in near future.

Takeaway lessons for future investigations

This review contributes key understandings and novel insights that may guide future work on *C. necator* H16 are as follows:

1. The remarkable progress of *C. necator H16* and its derivatives in the polymer field and biofuels is quite encouraging. The existence of versatile metabolic routes of 3-HA monomers is phenomenal and this organism has been a host for the various types of polymers containing monomers listed in Fig. 5. Supportively, none negative effects on the cell growth and PHA content were found in most of the engineering studies. However, some of the issues must be considered during strain selection for desired engineering approaches; H16Δ*phaA* strains contained partial reduction of 3-HB, whereas H16Δ*phaB* strains resulted in the drastic decline of 3-HB. The proof of concept can be found in the reported works (Mifune et al. 2010) (Budde et al. 2010, 2011). It must be considered that in H16Δ*phaB* mutants, the reduced reductase activity could hamper the regeneration of free CoAsh coming from PHA synthesis. The lesser activity affected cell growth resulting in less biomass and polymer (Jeon et al. 2014; Insomphun et al. 2015) (Table 2).
2. In the other hand, the mutants (H16Δ*phaC1P1* and H16Δ*phaC1*) have been appropriate for PHA production (Budde et al. 2011; Kawashima et al. 2015), whereas H16Δ*phaC*$_1$*AB*$_1$ mutant strain was found ideal for biofuels production and could be a platform for various building blocks (Crépin et al. 2016; Marc et al. 2017). Moreover, only limited combinations of mutant strains have been studied to date to establish the potential of *C. necator* H16. In particular, the multiple mutant strain (H16Δ*phaC*$_1$*AB*$_1$ and fatty acid oxidation) (Chen et al. 2015) and mutants like H16Δfatty acids oxidation pathway (Brigham et al. 2010) are underexplored and from our point of view, they hold considerable future in the challenge of incorporating new mcl-PHA monomers in the final polymer product.
3. The heterologous expression studies of synthases in mutants (H16Δ*phaC*$_1$, Re2133) as shown in Table. 2, cautioned that mcl-PHA accumulation in the given host cannot be limited by synthases, when the sufficient number of precursors is supplied. This idea is supported in the cases where the expression of PhaC$_{A.ca}$NSDG and PhaJ$_{A.ca}$ have greatly influenced extensive PHAs production (Table 2). Furthermore, handful of studies available to substantiate that the PHA content remained undisturbed and maintained in the modified mutants as compared with the wild type. This knowledge will prompt the use of various potent PHA synthases, which have been investigated to limited extent and holds great promises for additional studies in future.
4. From the studies, we could interpret that PhaA and PhaB copies are related with the high 3-HB polymer content, which is beyond the substrate specificity of the given PHA synthases. It has been evident in the expression studies of synthase enzymes belonging to *Pseudomonas* species in PHB-4 strain, where experiments noted the accumulation of only PHB on simple substrates (Timm et al. 1992; Timm and Steinbüchel 1992). This led us to ask whether the actual substrate specificity possessed by PhaC1$_{C.ne}$ enzyme is limiting the mcl-PHA polymer production in the native organism. Since, it has already been proven that PhaC$_{C.ne}$ enzyme have shown to polymerize the monomers of length ranging from C6–C10 (Dennis et al. 1998), especially when it was expressed in the host organism i.e., *E. coli* with appropriate channelling of mcl-PHA monomers towards it. Maybe, the absence of mcl-PHA monomer incorporation may not be attributed to the PhaC1$_{C.ne}$ enzyme and instead an adequate channelling will do. This may not be universally agreed and almost controversial to recent findings on the role of conserved regions among PHA synthases in the substrate specificity (Kim et al. 2017a). But, much more work could be performed to understand the fate and mutational effects of PhaC1$_{C.ne}$ synthase as well. Additionally, recent crystallographic studies revealed more structural information that could aid in the development of new engineering strategies (Kim et al. 2017a).
5. The contribution of PHA machinery proteins such as PhaB1, BktB in the reversal beta oxidation pathways must be highly appreciable and notable (Clomburg et al. 2018). The newly characterized SDR enzymes are also expected to have potential impact in the production of bulk chemicals (Magomedova et al. 2016). In addition, the conversion of industrial and oil wastes into valuable biofuels and biodegradable polymers had opened great opportunities towards sustainable waste management too. In this regard, the suitable use of crude glycerol

wastes in the production of potent fuels and chemicals is highly recommended.

6. Mutants - the future of PHAs: For decades, the deletion mutation strains were teaching researchers to understand the purpose of natural resources and their ability associated with the physiology of the given organisms. Alternatively, in this modern trend and evident in the case of *C. necator* H16, the researchers were inspired from obtained knowledge of mutants or variants and started treating them as potent tools for exploring potential applications. This is because, wild type organisms do not meet most of the requirements, as we have seen relatively compared with H16Δ$phaC_1AB_1$ and Re2133 strains. Evidently, the degree of investigation on the mutant strains have predominantly increased in the past years (Tables 1, 2). The fostering of mutants with the respective non-native pathway enzymes have endowed them with the ability to produce various materials (Table 2). We all apprehend the significance of these mutants in various occasions and how they have been adapted for several applications as stated earlier. In this context, mutants have been, and will continue to be, important. Hence, it is noteworthy to develop a collection bank for those strains, as mention in Table 1, to make them accessible to ongoing research(ers). Such initiative would be helpful to develop more novel ideas and put such potent mutants in practice.
7. On the outbreak of the new findings, we comprehend that many factors, include rational engineering of PHA synthases, adding fatty acid oxidation enzymes ($PhaJ_{A.ca}$, $PhaJ_{4aR.eu}$), replacement of phasins, developing novel mutants, have significantly improved polymer production in different degrees. Here, we envisage that a balanced engineering of all the possible factors together in a single pot will be necessary to achieve the goal of incorporating mcl-monomers. At the same time, there have been many other works in *E. coli* which can be easily adapted in *C. necator* H16. The most suitable example is the *E. coli* mutants of cell division machinery and their positivity towards PHA production (Jiang and Chen 2016).

Globally, we could say that *C. necator* H16 and its variants are able to demonstrate the ability to adapt to a wide variety of conditions, able to provide very broad substrate specificity enzymes, produce many different interesting metabolites, and frequently participate in different routes of where they could be adapted to specific applications. Such experiences from *C. necator* and their evolved strains can be directed to the subsequent researches that are focusing to produce mcl-PHAs.

Concluding remarks

This review presents a portrayal of the current state of knowledge on *C. necator* H16. The available data would be useful towards understanding its genome and their relationship to PHA production. Our analysis evidences its genetic diversity nature and customizable resources for numerous applications. Additionally, the newly developed mutants have fuelled innovative thoughts and gave *C. necator* H16 new perspectives on the production of biofuels and polymers. Although the metabolic network inhabited by this organism is abundant, yet difficulties to incorporate high carbon monomers numbers into the biopolymer prevail, becoming a central research for future investigations. This longstanding concern may be further enhanced by metabolic engineering, but also by better manipulation of synthases/Phasins and the control of the fermentation processes. Substantial advances in the PHA field could emerge from an increased understanding of the mutants' properties and how to engineer them with the appropriate non-native pathways. With this accumulative knowledge, the continual synthesis of beneficial biofuels and bulk chemicals can be anticipated.

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