Synthetic biology toolkit for engineering *Cupriviadus necator* H16 as a platform for CO$_2$ valorization

Haojie Pan[1], Jia Wang[1], Haoliang Wu[1], Zhongjian Li[1] and Jiazhang Lian[1,2]*

Abstract

Background: CO$_2$ valorization is one of the effective methods to solve current environmental and energy problems, in which microbial electrosynthesis (MES) system has proved feasible and efficient. *Cupriviadus necator* (*Ralstonia eutropha*) H16, a model chemolithoautotroph, is a microbe of choice for CO$_2$ conversion, especially with the ability to be employed in MES due to the presence of genes encoding [NiFe]-hydrogenases and all the Calvin–Benson–Basham cycle enzymes. The CO$_2$ valorization strategy will make sense because the required hydrogen can be produced from renewable electricity independently of fossil fuels.

Main body: In this review, synthetic biology toolkit for *C. necator* H16, including genetic engineering vectors, heterologous gene expression elements, platform strain and genome engineering, and transformation strategies, is firstly summarized. Then, the review discusses how to apply these tools to make *C. necator* H16 an efficient cell factory for converting CO$_2$ to value-added products, with the examples of alcohols, fatty acids, and terpenoids. The review is concluded with the limitation of current genetic tools and perspectives on the development of more efficient and convenient methods as well as the extensive applications of *C. necator* H16.

Conclusions: Great progress has been made on genetic engineering toolkit and synthetic biology applications of *C. necator* H16. Nevertheless, more efforts are expected in the near future to engineer *C. necator* H16 as efficient cell factories for the conversion of CO$_2$ to value-added products.

Keywords: *Cupriviadus necator* H16, *Ralstonia eutropha* H16, Synthetic biology, Metabolic engineering, CO$_2$ conversion, Biomanufacturing

Background

With increasing concerns on climate change and sustainability, new concepts such as "Circular Economies" and "Carbon Neutrality" have been proposed to call for the production of chemicals and biofuels from renewable feedstocks [1, 2]. However, corn-based biofuel production has triggered a fierce debate on "Food versus Fuel"

[3] and the second-generation biofuels from lignocellulose biomass still suffer from low efficiency and high cost [4]. Alternatively, CO$_2$ is generally considered as the third-generation feedstock for biofuels [5]. The realization of CO$_2$ recycling will be an effective way to address current challenges in energy, resource, and environment. Currently, many efforts have been devoted to establishing efficient CO$_2$ conversion systems. On one hand, chemists have been designing and engineering new catalysts for converting CO$_2$ to fuels such as CO, CH$_4$, and CH$_3$OH with high energy efficiency, although the product portfolio is expected to be further expanded [6, 7]. On the other hand, via metabolic engineering of photosynthetic

*Correspondence: jzlian@zju.edu.cn
[1] Key Laboratory of Biomass Chemical Engineering of Ministry of Education, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China
Full list of author information is available at the end of the article

Pan *et al. Biotechnol Biofuels* (2021) 14:212

Page 2 of 20

microorganisms such as cyanobacteria, biologists have realized the direct conversion of CO_2 to various value-added products [8]. In recent years, microbial electrosynthesis (MES) system, coupling electrocatalysis with microorganisms, is a feasible strategy that can be more efficient than natural photosynthesis for converting CO_2 to complex and high-value products [9, 10]. In MES, CO_2 is fixed and reduced by microbial cells and the required redox equivalents, such as formate, H_2, and electrons, are provided by electrochemical reactions [11, 12].
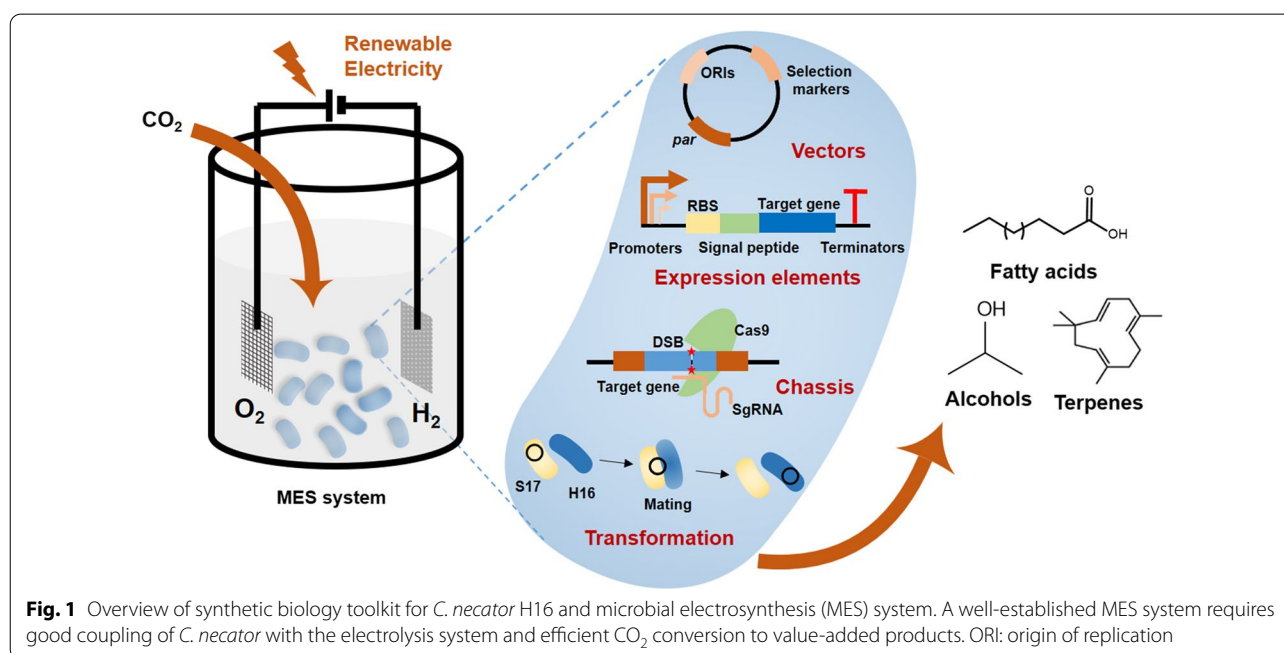
Cupriviadus necator H16, also known as *Hydrogenomonas* H16, *Alcaligenes eutrophus* H16, *Wautersia eutropha* H16, and *Ralstonia eutropha* H16, is a Gram-negative betaproteobacterium discovered 60 years ago [13–16]. It has gained intensive research interests in recent years due to its capability of CO_2 fixation and conversion, especially in MES [10, 11, 17–26]. *C. necator* represents a model facultative chemolithoautotroph that can utilize fructose, gluconate, various organic acid, and CO_2 as carbon sources. While carbohydrates are metabolized via the Entner–Doudoroff (ED) Pathway with 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda) as the key enzyme, *C. necator* H16 also embraces all the necessary genes of the entire Calvin–Benson–Basham (CBB) cycle. Besides, two oxygen-tolerant [NiFe]-hydrogenases enable *C. necator* H16 to power the reduction of CO_2 by hydrogen [27–30]. Another research hotspot about *C. necator* H16 is polyhydroxyalkanoate (PHA) production, which is a kind of biodegradable materials with high thermoplasticity, elasticity, and biocompatibility. PHA biosynthesis has been extensively reviewed and will not be discussed here [31, 32]. While the carbon source utilization range [33] and the product scopes [34] of *C. necator* H16 have been reviewed elsewhere, the review is mainly focused on the development of synthetic biology tools. Complete genomic information [35–37] has laid the foundation for the development of these tools, and transcriptomics, proteomics, and metabolomics studies have provided the guidance for metabolic engineering [38–44].

In this review, synthetic biology toolkit available for *C. necator* H16 is firstly summarized, whose applications in engineering *C. necator* H16 as efficient cell factories for converting CO_2 to value-added products is followed for discussion (Fig. 1). The review is concluded with the limitations of current genetic tools and perspectives on extensive applications of *C. necator* H16.

Genetic engineering vectors

Vectors, based on either episomal plasmids or chromosomal insertion, introduce and stabilize heterologous genes in recipient cells. Up to now, various vectors have been established in *C. necator*, including autonomous replication elements for episomal plasmids and integration sites for chromosomal insertion [45–47]. The former is easy to control the copy numbers but suffers from serious plasmid loss, while the latter has high stability but low copy numbers. Therefore, how to choose appropriate expression vectors according to the target products is a worth-thinking and open-to-discussion question. Luckily, systems for plasmid stability and maintenance developed in the past few years offer more options for metabolic engineering [46, 48–52].

Fig. 1 Overview of synthetic biology toolkit for *C. necator* H16 and microbial electrosynthesis (MES) system. A well-established MES system requires good coupling of *C. necator* with the electrolysis system and efficient CO_2 conversion to value-added products. ORI: origin of replication

Pan *et al. Biotechnol Biofuels* (2021) 14:212

Page 3 of 20

Episomal plasmids

According to different replication mechanisms, plasmids can be assigned to different incompatibility groups. Most of incompatibility groups commonly used in Gram-negative bacteria are adopted successfully in *C. necator*: IncQ-group (e.g., RSF1010 [53, 54] and pJRD derived from RSF1010 [55]), IncP-group (e.g., RP4 (RK2) [56]), IncW-group (e.g., pSa [46]), and pBBR1 [57]. pBBR1 is the most commonly used in *C. necator*. In addition, the plasmid, pCUP3, harboring the plasmid partition and replication region of the mega-plasmid pMOL28 from *Ralstonia metallidurans* CH43, shows effectivity in *C. necator* H16 and compatibility with the native plasmid, pHG1 [48]. The co-existence of plasmids from different incompatibility groups in one cell was found to be feasible. Li et al. achieved autotrophic production of fatty acids using plasmids based on pBBR1 and RP4 [58] and Claassens et al. replaced the CBB cycle with the reductive glycine pathway using plasmids based on RP4 and RSF1010 [59].

A properly regulated replication system, including cis-region (e.g., origin of replication, ORI) and trans-region (e.g., replication proteins), largely determined the copy numbers and stability during cell division [45]. Plasmids with different ORIs and accordingly different copy numbers are listed in Table 1. It is noteworthy that copy numbers of the RP4-based plasmids (e.g., pCM) are regulated by the *trfA* gene [60] and site-directed mutagenesis of *trfA* could increase the plasmid copy numbers to even higher than 40 per cell [47]. As for the plasmid stability, no comparative study has been conducted under the same condition. Generally, all the episomal plasmids suffer from segregational instability. Taking the RSF1010-based plasmid for example, even in the presence of chloramphenicol, the percentage of plasmid-bearing cells

was reduced to 10% after 70 h of fermentation [54]. As for the pBBR1-based plasmid, about 95% and 80% were lost in minimal medium (MM) and LB after four subcultures without antibiotic pressure, respectively [61]. RP4-derived plasmids are considered as the most stable in *C. necator*. However, only about 70% of the cells carrying RP4-based plasmid cultured in the tetracycline-free medium were tetracycline resistant compared to those cultured with tetracycline after 24 h of fermentation [49].

Elements for plasmid stability and maintenance

A common strategy for plasmid maintenance is to keep the selection pressure, such as the addition of antibiotics to the medium. Three antibiotics have been found to work as screening pressure in *C. necator* (Table 2): kanamycin, tetracycline, and chloramphenicol. The working concentration to some extent is dependent on the culture conditions and drug manufacturers. However, antibiotic supplementation is not a good choice for large-scale fermentation due to its financial expenses and negative environmental impact [62]. In addition, antibiotic selection suffers from low stability. The study of Voss et al. showed that 38% of the tetracycline-resistant and 62% of the kanamycin-resistant cells lost their plasmids during cultivation [52]. To address the limitation of antibiotics, two additional strategies have been employed: toxin/anti-toxin system and metabolism-based plasmid addiction system.

Toxin/anti-toxin is a mechanism to regulate cell growth and death under various stress conditions in bacteria and archaea. These toxins are highly stable and may inhibit cell growth or even cause cell death by blocking essential cellular processes, whereas cognate anti-toxins can relieve such toxicity but continuous expression

Table 1 Genetic engineering vectors commonly used in *C. necator*

Vectors	Description	Reference
Episomal plasmids		
pCM	RP4 ORI, low copy, ~8	[47]
pCM271	RP4 ORI, mutation in *trfA*, medium copy, ~40	[47]
pBBR1	medium copy, ~40	[57]
pKT230	RSF1010 ORI, medium copy, ~10	[47]
pSa	low copy, 2~3	[46]
pCUP3	pMOL28 ORI, low copy, 1	[48]
Integrative plasmids		
pJQPPCm	*phaP* (Phasin) as integration locus	[53, 54]
pJV7	*phaC1* (PHA synthase) as integration locus	[51]
pCB42	*phaB1* (acetoacetyl-CoA reductase) as integration locus	[66]
pLH63	*phaB2C2* as integration locus	[26]
pEX100T	*ldh* (L-lactate dehydrogenase) as integration locus	[52]
pLO1	*norR2A2B2* (NO reductase) as integration locus	[49, 68]

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 4 of 20

Table 2 Screening markers available in *C. necator*

Marker	Description	Reference
Resistance marker		
Neo^R/Kan^R	Resistant to kanamycin; 200–350 μg/mL	[47]
Tc^R	Resistant to tetracycline; 10–25 μg/mL	[47]
Cm^R	Resistant to chloramphenicol; 25–50 μg/mL	[47]
Nutrition-deficient complementary marker		
hoxABCJ	*C. necator*Δ*hoxA*; transcription regulator of two [NiFe]-Hydrogenases	[49]
eda	*C. necator*Δ*eda*; key enzyme of ED pathway	[52]
xfp	*C. necator*Δ*eda*; key enzyme of the alternative phosphoketolase-dependent pathway	[50]
proC	*C. necator* Δ*proC*; indispensable gene for proline biosynthesis	[51]
Counter-selectable marker		
sacB	Lethal in sucrose-containing medium	[114]

The antibiotic ranges to some extent is dependent on the culture conditions and drug manufacturers

is required due to low stability. Thus, once the plasmid bearing the toxin/anti-toxin operon was lost, previously expressed and stably retained toxins would kill or inhibit the growth of the plasmid-free cells [63]. The pMOL28-derived parABS28 is such a system that shows decent performance for plasmid partition and maintenance. Sato et al. showed nearly no plasmid loss due to this post-segregational killing system [48]. RP4 partitioning system from natural plasmid RP4 (RK2) also contains toxin/anti-toxin system (i.e., parDE) [64]. Gruber et al. combined RP4 partition sequence with different ORIs (PR4, RSF1010, pBBR1, and pSa) and obtained highly stable plasmids independent of the replication system [46]. The studies done by Sydow et al. [61] and Krieg et al. [19] further demonstrated the effectiveness of this strategy.

The metabolism-based plasmid addiction system is comparable to the nutrition-deficient complementary system in yeast. As mentioned above, *eda* encodes a key enzyme for carbohydrate metabolism in *C. necator*. Voss et al. knocked out *eda* gene in the host and constructed pBBR1MCS-2 bearing complementary *eda* to produce cyanophycin. 93% of the cells maintained the plasmids without antibiotic selection and the production was at least fivefold higher [52]. The phosphoketolase-dependent pathway (the key gene is *xfp*) is an alternative way to restore the capability of fructose utilization. Fleige et al. heterologously expressed *xfp* gene from *Bifidobacterium animalis* on the plasmids in the *eda*-deficient *C. necator* strain to obtain a stable production platform [50]. A similar system was established based on the pyrroline-5-carboxylate reductase gene (*proC*), an indispensable gene for proline biosynthesis, which was useful in MM where proline is not supplemented [51]. Lutte et al. complemented the deficiency in the native hydrogenase transcription factor *hoxA* with *hoxABCJ* from *Alcaligenes sp.* M50 on the plasmids to construct a novel addiction system under lithoautotrophic conditions, which showed no plasmid loss and higher production of cyanophycin [49] (Table 2).

Genome integration vectors

As high copy number plasmids usually exert metabolic burdens on cell growth, integration into the chromosome is preferred. Target gene expression cassettes are generally cloned between two ~ 500 bp homologous arms and integrated into specific genome sites [49, 52, 65]. Commonly used integration sites in *C. necator* are listed in Table 1. Noteworthy, most of integration sites are related to the polyhydroxybutyrate (PHB) biosynthetic pathway, which is one of the most thoroughly studied metabolic pathways and whose disruption can easily redirect carbon flux to produce the target products. Srinivasan et al. integrated P_{phaP}-*OPH* into the *phaP* site and achieved soluble and functional expression of organophosphohydrolase, a protein prone to form inclusion body in *Escherichia coli* [53]. Budde et al. replaced *phaB1* and *phaC1* with *phaB* and *phaC* homologs, respectively, to investigate their functions [51, 66]. Li et al. integrated part of the isobutanol and 3-methyl-1-butanol biosynthetic pathway into the *phaB2C2* site, with the remaining genes cloned on a plasmid, and obtained the production of higher branched-chain alcohols with a titer of over 1.4 g/L in an MES system [26]. Besides that, the lactate dehydrogenase gene (*ldh*) is another commonly used integration site, whose deletion will reduce the formation of lactate especially under restricted oxygen conditions. Voss et al. integrated *cphA* (cyanophycin synthetase) into the *ldh* site for the biosynthesis of cyanophycin [52]. Similarly, Lutte et al. integrated *cphA* into the *norR2A2B2* site [49], encoding NO reductase, an enzyme involved in the denitrification pathway [67, 68]. Currently, genome integration has not been used as commonly as episomal plasmids due to the low gene dosage, limited integration sites,

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 5 of 20

and lack of efficient integration tools. Although multiple-copy gene integration of P$_{phaP}$-*OPH* done by Srinivasan et al. showed high stability, the production was still lower than that of the plasmid-based system [54]. Nevertheless, with the development of synthetic biology, genome integration, as a complementary approach to plasmid, will play increasingly important roles in biotechnological applications. Thus, it is expected that genome integration tools and more stable integration sites will be established and characterized in the near future, which will be further discussed in the "Genome editing" Section.

Heterologous gene expression elements

A precise expression system, consisting of constitutive or inducible promoters, 5′-untranslated regions (UTRs), signal peptides, target genes, and terminators, is essential for constructing cell factories. To balance cell growth and product biosynthesis, a well-tuned expression system is highly desirable, which mainly depends on diverse elements determining transcription efficiency (e.g., promoters and terminators) and translation efficiency (e.g., ribosomal binding sites, RBS). In this section, these available elements in *C. necator* will be summarized.

Constitutive and inducible promoters

In recent years, numerous well-characterized and controllable promoters have been developed, facilitating the applications in PHB synthesis and CO_2 conversion. Native constitutive promoters related to PHB synthesis (P$_{phaC1}$), pyruvate metabolism (P$_{pdhE}$) [69], acetyl-CoA synthesis (P$_{acoE}$) [70], and translation (P$_{rrsC}$) [71] are employed in metabolic engineering. Among them, P$_{phaC1}$ is the most commonly used [26, 59]. Unfortunately, these native promoters are relatively weak. Promoters derived from other organisms can be used to extend the promoter strength range. Notably, P$_{lac}$ and its derivatives from *E. coli* can work as constitutive promoters in *C. necator* due to the absence of *lacI* and *lacY* homologs in the genome. Fukui et al. showed P$_{tac}$ exhibited 1.5- to 2-fold higher GFP expression than that of P$_{phaC1}$ [72]. Arikawa et al. compared the strength of various promoters (P$_{trc}$ ≥ P$_{lacUV5}$ > P$_{trp}$) and P$_{trc}$ was at least 20-fold higher than P$_{phaC1}$ [73]. Although the absolute value may vary in different laboratories due to subtle differences in promoter architecture, cultivation conditions, and analytical devices, P$_{trc}$ and P$_{tac}$ (a mutant of P$_{trc}$ that can initiate transcription more efficiently without the presence of catabolite activator protein, CAP) are relatively strong promoters in *C. necator*. Subsequently, Gruber et al. tested a series of promoters derived from bacteriophage T5 and P$_{j5}$ was identified as the strongest promoter, about fivefold higher than P$_{tac}$ [46]. To get promoters with a broad activity range, Li et al. constructed a P$_{phaC1}$ promoter library by mutating the last four nucleotides of the -35 region, and obtained a variety of promoters weaker than the native P$_{phaC1}$ [71]. Alagesan et al. combined core sequences of previously characterized promoters with upstream and downstream insulation sequences to construct a promoter library, in which four promoter variants were identified to be stronger than P$_{j5}$ [74]. Johnson et al. adopted rational engineering approaches of point mutation, length alteration, incorporation of regulatory genetic element, promoter hybridization, and configuration alteration, and obtained a 42-promoter library displaying a wide range of activities based on P$_{phaC1}$, P$_{rrsC}$, P$_{j5}$, and P$_{g25}$ [75].

For more precise regulation, inducible promoters are preferred, particularly those derived from other organisms and synthetic systems, because of their orthogonality to *C. necator* and efficiency for directing carbon flux to the biosynthesis of the target products. AraC/ P$_{araBAD}$ from *E. coli* is the most widely used and exhibits the strongest transcription activity. Nonetheless, leaky expression and growth defects limit its further application [76, 77]. RhaRS/P$_{rhaBAD}$, also from *E. coli*, seems to be a more promising regulatory system. Although the maximum induction level of RhaRS/P$_{rhaBAD}$ is slightly lower than that of AraC/P$_{araBAD}$, the induction ratio is much higher due to lower leaky expression [74]. Sydow et al. showed that the growth of *C. necator* was virtually not altered even at the highest expression level (i.e., 11 mM L-rhamnose) [61]. Modified LacI/P$_{lac}$ system can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) when lactose permease (*lacY*) was co-expressed. Bi et al. constructed pYI-UV5Trfp, containing *lacY* and *lacI*, which was found to be functional in *C. necator* although its expression level was relatively low [47]. Hanko et al. demonstrated that MmsR/P$_{mmsA}$ and HpdR/P$_{hpdH}$ derived from *Pseudomonas putida* were highly inducible systems with 3-hydroxypropionic acid as both the inducer and carbon source [78]. PM/P$_{xyls}$-m-toluic acid [47], AcuR/ P$_{acuRI}$-acrylate [74], and YpItcR/P$_{ccl}$-itaconate [79] systems were found to work in *C. necator* as well in spite of high background expression levels. Synthetic gene circuits, which combine those characterized elements, can be constructed to further develop subtler inducible expression systems. Gruber et al. combined constitutive promoter P$_{j5}$ with lac and cumate regulatory elements to construct LacI/P$_{j5-lac}$ and CymR/P$_{j5-cmt}$. CymR/P$_{j5-cmt}$ was determined to be better because of lower leaky expression, slighter growth defects, and cheaper inducer [65]. Li et al. developed a synthetic anhydrotetracycline-controllable gene expression system TetR/P$_{rrsC-tetO}$ by stepwise optimization of the type of *tetO* (*tetO1* and *tetO2*), the copy number of *tetO1*,

Pan *et al. Biotechnol Biofuels* (2021) 14:212

Page 6 of 20

and the expression level of *tetR* [71]. Similarly, Aboulnaga et al. constructed a TetR/P$_{tolC-tetO}$ system with a high induction ratio [80]. Barnard et al. integrated T7 RNA polymerase gene under the control of P$_{phaP}$ and used T7 promoter to achieve high-level recombinant protein expression just like pET system, the most widely used expression system in *E. coli* [81]. Bi et al. adopted a similar strategy to couple AraC/P$_{araBAD}$ with T7 system [82]. Inducible promoters developed in recent years are summarized in Table 3, many of which are found to be orthogonal to each other. For instance, the addition of L-arabinose has no impact on RhaRS/P$_{rhaBAD}$ and HpdR/P$_{hpdH}$ [78].

Although inducible promoters are effective for precise control of gene expression levels, inducer implementation significantly increases the cost of the whole bioprocess and limits their practical applications. On the contrary, some native promoters related to essential metabolism can implement auto-induction without the need of inducers. P$_{phaP}$ is activated under phosphate limitation conditions, tightly coupling with PHB biosynthesis [53, 72, 83]. P$_{cbbL}$ is induced under chemolithoautotrophic conditions and repressed on pyruvate and fructose [49, 84–86]. P$_{acoD}$ and P$_{acoX}$, related to the acetoin metabolism, are induced by acetoin and commonly used for metabolic engineering, although these two native promoters are rather weak [69, 87]. P$_{SH}$ and P$_{MBH}$ (hydrogenase promoters) are probably the strongest native ones identified so far in *C. necator*, which are induced on glycerol and repressed on fructose [88–90]. However, applications of these auto-inducible promoters, such as dynamic control, have not been fully explored yet. Overall, to deal with complex metabolic engineering tasks, more controllable and elaborate promoter systems should be constructed and tested. In addition to classical promoters, riboswitches that can precisely control the translation initiation rate can be explored in *C. necator* [91, 92].

5′-Untranslated regions (UTRs)

Translation efficiency mainly depends on the stability of mRNA and its ability to bind to ribosomes. Thus, the key to efficient translation is a suitable RBS, regardless of whether it is native, derived from *E. coli* or synthetically designed. Based on an RBS calculator developed by Voigt group [93], Alagesan et al. built an RBS library in *C. necator* with variable strengths, exhibiting more than a tenfold dynamic range [74]. They further verified that RBS strength could vary dramatically relying on the sequences of the promoters and target genes [94]. Therefore, in order to build a well-tuned expression system, a range of RBS should be individually evaluated in *C. necator*. In addition, T7 stem–loop structures and A/U-rich sequences can be employed to increase the stability of mRNA. Bi et al. added a T7 stem–loop structure between P$_{araBAD}$ and RBS sequence, resulting in a twofold increase in the expression of RFP [47]. Alagesan et al. further demonstrated that T7 stem–loop structures could universally enhance gene expression in *C. necator*, while A/U-rich sequences could alter and fine-tune gene expression levels [74].

Signal peptides

Signal peptides are not always needed, but can play crucial roles in some cases, such as the production of recombinant proteins. Secretion of target proteins into the culture broth or periplasmic space will not only save the cost of product recovery and purification, but also promote correct folding of the target proteins due to the specific oxidation–deoxidation environment. For

Table 3 Comparison of inducible promoters developed in *C. necator*

Promoter	Inducer and concentration	Induction ratio	Effects on growth
AraC/P$_{araBAD}$	10 mM L-arabinose	1200	Inhibited cell growth; not consumed
RhaRS/P$_{rhaBAD}$	11 mM L-rhamnose	1960	Virtually not altered cell growth; not consumed
LacI/P$_{lac}$	1 mM IPTG	~300	Inhibited cell growth at high concentration; not consumed
PM/P$_{xylS}$	1 mM m-toluic acid	~300	–
AcuR/P$_{acuRI}$	5 mM acrylate	33	Consumed
YpItcR/P$_{ccl}$	5 mM itaconate	105	Not consumed
MmsR/P$_{mmsA}$	10 mM 3-hydroxypropionic acid	51.5	Growth-retarding effect at the beginning of cultivation; consumed
HpdR/P$_{hpdH}$	10 mM 3-hydroxypropionic acid	516.6	Growth-retarding effect at the beginning of cultivation; consumed
LacI/P$_{j5-lac}$	1 mM IPTG	~7	Growth inhibition at high concentration; not consumed
CymR/P$_{j5-cmt}$	120 µM p-cumate	22	Nearly no effect on growth
TetR/P$_{rrsC-tetO}$	200 ng/mL anhydrotetracycline	~1100	No negative effect on growth; not consumed; antibiotic
TetR/P$_{tolC-tetO}$	25 ng/mL doxycycline	~180	No negative effect on growth; not consumed; antibiotic

The induction ratio is recalculated based on AraC/P$_{araBAD}$ as a benchmark according to references [47, 65, 71, 74, 78–80]

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 7 of 20

instance, the signal peptide (i.e., a Sec signal peptide) of Caa, the periplasmic carbonic anhydrase, has the capability of secreting proteins to the periplasm [95]. Membrane-bound [NiFe]-hydrogenase (MBH) attaches to the plasma membrane and functions at periplasm, whose signal peptide (i.e., a Tat signal peptide) is located at the N-terminus of the small subunit HoxK [87]. Such signal peptides will be valuable for enzyme relocation for the assembly of complex metabolic networks. Recently, Tang et al. relocated VHb, an oxygen carrier, to the periplasm by the traction of MBH signal peptide to promote cell growth and PHB synthesis under oxygen-limiting conditions [96]. Unfortunately, only a limited number of signal peptides in *C. necator* have been characterized for synthetic biology applications and yet to be explored in the near future.

Besides the secretion of the target proteins, signal peptides can be employed for protein immobilization or pathway compartmentalization, by taking advantage of the accumulation of PHB granules in *C. necator*. The PHB-associated proteins such as PHB synthases (PhaC) [97], PHB depolymerases (PhaZ), and phasins (PhaP) [98], which are attached to the surface of PHB particles, can be used as anchor proteins. Barnard et al. employed fusion expression with phasins (PhaP) to attach the target proteins to the granules of PHB, leading to the formation of an "affinity matrix" in *C. necator* and simplified downstream purification steps [98]. Such application has not been fully explored in *C. necator*, but widely used in *E. coli* [99, 100]. Wong et al. constructed modular polyhydroxyalkanoate scaffolds for protein immobilization by fusing SpyCatcher protein with PHB synthases (PhaC) and fusing SpyTag with the target proteins [100]. A similar protein immobilization strategy can be adopted in *C. necator*, and it is expected that the co-location of multiple proteins on the PHB scaffolds (pathway compartmentalization) can be established in the near future for metabolic engineering applications [101].

Target genes

Either for efficient production of foreign proteins or successful construction of long metabolic pathways, selection of endogenous or exogenous genes from suitable organisms is important. Codon optimization is an effective strategy for efficient expression of heterologous genes, because the GC content of *C. necator* genome is about 66% and much higher than that of most organisms [36, 102, 103]. Differences in GC content may impair transcriptional and translational efficiencies, limiting the enzyme activities and thus production yields. Grousseau et al. performed codon optimization of *adc* and *adh* from *Clostridium* species (about 30% GC), resulting in an 8.9 ± 3.0-fold increase in isopropanol production [103].

Another strategy for improving enzyme activities is to increase the copy number of the rate-limiting enzyme encoding genes, which is quite useful in breaking the "bottleneck" of long pathway. In the same case of isopropanol production, a second copy of *adh* increased the yield for additional 1.20 ± 0.18-fold [103].
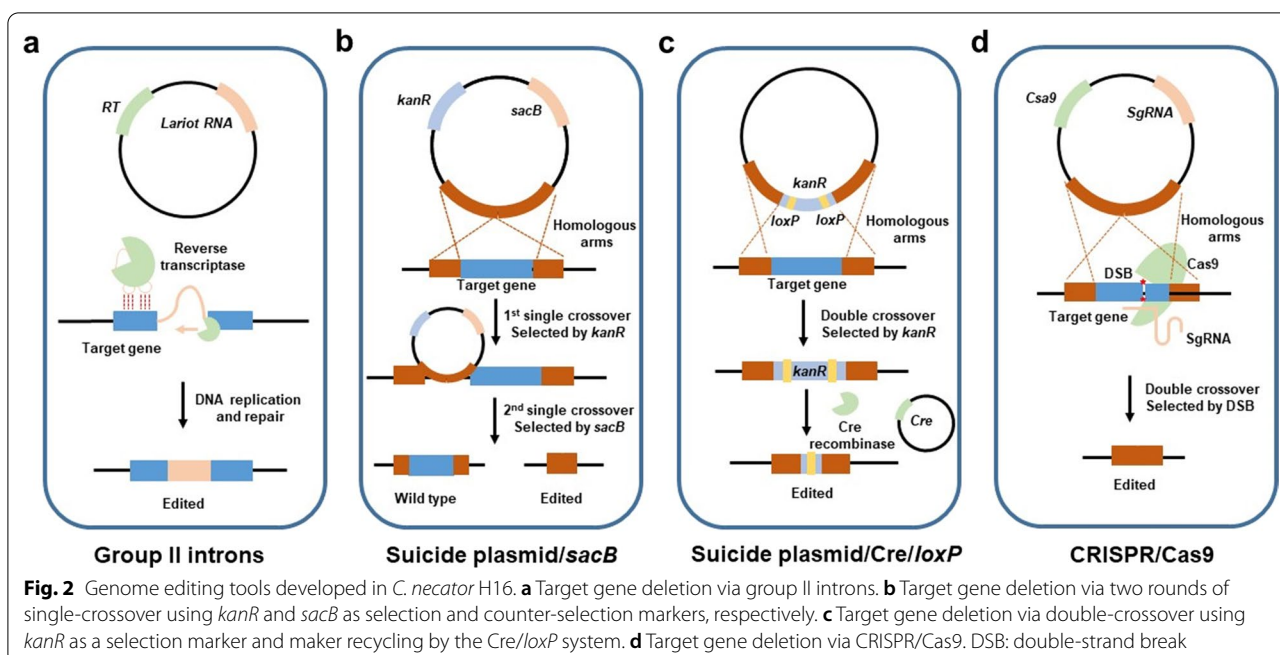
Terminators

A transcriptional terminator is important for avoiding massive energy waste for the production of unnecessary transcripts and the formation of undesirable secondary structures in a few cases [104]. However, the contribution of terminators to recombinant protein production and metabolic engineering applications is largely overlooked. To date, available terminators in *C. necator* are still rather limited, including pTOPO Terminator [54], *rrnB* T1 Terminator [105], *rrnD* T1 Terminator, and T7Te Terminator [80]. The *E. coli* derived *rrnB* T1 Terminator is the most commonly used [105]. Bi et al. used a dual-terminator (*rrnB* T1 + T7Te Terminator) to ensure transcription termination [47]. Aboulnaga et al. compared the bi-direction T7 Terminator with mini-*rrnD* T1 Terminator and found the latter to have a better termination efficiency [80].

Platform strain and genome engineering

In order to construct robust and efficient microbial cell factories, platform strains should be modified via genome editing tools. In recent years, several *C. necator* hosts, such as for cultivating in an MES system or producing fatty acid derivatives, have been established. Limited by genome editing tools and basic metabolic knowledge of *C. necator*, adaptive evolution offers a powerful alternative for chassis engineering.

Genome editing

Before the advent of new genome editing tools, UV mutagenesis [106] and chemical mutagenesis [107] played a crucial role in constructing many *C. necator* mutants still in use today. Tn5 transposon is another powerful tool in *C. necator* for random integration into the chromosome and it is superior to previous methods as it mainly causes single-gene mutations [108]. Peoples et al. constructed a PHB-negative mutant based on the insertion of Tn5 into the *phaC* site [109]. Barnard et al. achieved the integration of *OPH* expression cassette via Tn5 transposon [81]. Different from random integration based gene disruption, Park et al. developed a targeted gene knock-out system RalsTron, based on the group II introns. The functional genes were disrupted by inserting introns into specific gene loci, based on a mechanism named retrohoming (Fig. 2a) [110].

Pan *et al. Biotechnol Biofuels* (2021) 14:212

Page 8 of 20

Fig. 2 Genome editing tools developed in *C. necator* H16. **a** Target gene deletion via group II introns. **b** Target gene deletion via two rounds of single-crossover using *kanR* and *sacB* as selection and counter-selection markers, respectively. **c** Target gene deletion via double-crossover using *kanR* as a selection marker and maker recycling by the Cre/*loxP* system. **d** Target gene deletion via CRISPR/Cas9. DSB: double-strand break

Homologous recombination (HR) is the most commonly employed strategy for gene disruption and insertion. Due to the relatively low HR efficiency, suicide plasmids have been constructed to greatly promote genome editing of *C. necator*, including pKNOCK (R6K ORI) [111], pLO1 (ColEl ORI) [112], pJQ200mp18 (P15A ORI) [113], and pK18mobsacB (pMB1 ORI) [114]. As their ORIs cannot replicate in bacteria other than enterobacteria, they function as suicide plasmids in *C. necator* and integrate into the chromosome via HR. Counterselectable marker *sacB* (Table 2) and Cre/*loxP* system are employed to recycle the resistance marker for multiround operation. As *sacB* (from *Bacillus subtilis*) encodes levansucrase that catalyzes the conversion of sucrose to levan, a cytotoxin, sucrose-containing medium can be used to select plasmid backbone free cells through a second-round single-crossover (Fig. 2b) [29, 113, 114]. Cre (from phage P1) is a kind of site-specific recombinase, specifically recognizing *loxP* sites and promoting HR between two *loxP* sites [115]. Gruber et al. integrated *lacY* into the *phaC* site by double-crossover and removed the chloramphenicol resistance marker using the Cre/*loxP* system (Fig. 2c) [65].

However, these tools suffer from low efficiency and being time-consuming. Knockout efficiency using RalsTron was only 12.5% in the study of Park et al. [110]. Although suicide plasmid-based system can perform knock-out and knock-in, two rounds of operation are required and the efficiency is still not satisfactory. The emergence of CRISPR/Cas technique provides more

effective and time-saving tools. A single guide RNA (sgRNA) guides the Cas protein to bind and cut DNA specifically, resulting in the formation of a double-strand break (DSB), which is repaired by non-homologous recombination end joining (NHEJ) or HR to achieve gene knock-out or knock-in [116]. Xiong et al. was the first to apply CRISPR/Cas9 in *C. necator* and assembled three elements, sgRNA, Cas9, and donor DNA in one plasmid with editing efficiencies ranging from 78.3 to 100% (Fig. 2d) [117]. Due to the difficulties in transforming large plasmids to *C. necator*, the integration of large DNA fragments based on the CRISPR/Cas9 system has not been achieved yet. For synthetic biology applications of *C. necator*, more powerful genome engineering tools, e.g., multiplex genome editing technique either based on CRISPR/Cas9 or base editor [118, 119], should be developed in the near future.

Adaptive evolution

Adaptive evolution is a powerful tool to engineer *C. necator* with complex phenotypes, such as high tolerance or high utilization rate of carbon sources, due to our limited knowledge of the metabolic and regulatory network. With the development of sequencing and omics technology, an in-depth understanding of genotype and phenotype relationships becomes possible. Liu et al. built a water-splitting biosynthetic system with the need for ROS (reactive oxygen species)-resistant variants of *C. necator*. *C. necator* was exposed to the water-splitting system for 11 consecutive days and sequencing analysis

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 9 of 20

showed that mutations in the membrane protein related to cation/multidrug efflux system and transcriptional regulator contributed to ROS resistance [10]. Gonzalez-Villanueva et al. obtained *C. necator* 16 variant v6C6 with a specific growth rate in glycerol 9.5 times faster than the wild-type strain via adaptive laboratory evolution, and identified glycerol kinase as the key enzyme for improved glycerol utilization [120]. Claassens et al. combined short-term evolution and rational engineering, achieving CO_2 assimilation via more efficient reductive glycine pathway other than the endogenous CBB cycle [59]. With the aid of synthetic biology tools, the combination of rational design and laboratory adaptive evolution is expected to play an increasingly important role in the construction and optimization of *C. necator* cell factories in the near future.

Commonly used *C. necator* platform strains

The most commonly used *C. necator* host is the PHB-negative mutant (Table 4), in which carbon flow can be easily redirected to the synthesis of the target products. The first such mutant (H16PHB⁻4) was constructed by Schlegel et al. through 1-nitroso-3-nitro-l-methylguanidine (NMG) treatment [107]. Unfortunately, due to random mutagenesis, defects in fatty acid metabolism and regulation of CBB cycle were observed in H16PHB⁻4 [43]. Subsequently, H16Δ*phaCAB* and H16Δ*phaC1* were precisely constructed via targeted genome editing tools [83, 121]. In spite of the presence of multiple orthologues, the deletion of the main operon *phaCAB* or the key synthase gene *phaC1* nearly abolishes the capability of PHB biosynthesis in *C. necator*.

Another effort was devoted to reversing the inability to utilize glucose. Schlegel et al. constructed H16 G⁺1 via UV mutagenesis [106] and Raberg et al. subsequently obtained a glucose-utilizing mutant (H16Δ*nagRnagE*Ala153Thr) via rational engineering [122, 123]. In recent years, more platform strains have been constructed to cater to the metabolic engineering needs for the biosynthesis of different products. For instance, Brigham et al. deleted two native β-oxidation operons in *C. necator*, which is suitable for the production of fatty acid-derived fuels and chemicals [124]. Besides these driven by direct metabolic engineering applications, platform strains for more efficient genetic manipulation have been established as well. Xiong et al. constructed a platform strain, C5, for electroporation by deleting putative restriction modification (RM) genes *H16_A0006* and *H16_A0008-9* [117].

Transformation methods

Regardless of the expression of recombinant proteins, construction of exogenous pathways, or genome editing of *C. necator*, foreign DNA should be introduced into the host cell first. Thus, DNA transformation is a fundamental technique for synthetic biology. The transformation efficiency of non-model Gram-negative bacteria is usually low probably due to the complicated cell envelope structures. Thus, heat-shock-based chemical transformation is not feasible and conjugation and electroporation are commonly employed in *C. necator*. The efficiency of conjugation and electroporation is related to plasmid size and stability, transformation condition, and the host [125]. Notably, Sato et al. showed that the transformation efficiency of plasmid with the maintenance element par-ABS28 derived from pMOL28 was 500-fold higher [48].

Conjugation

Conjugation is the process of transferring genetic materials between two bacteria via cell mating, whose advantage lies in generality, i.e., it is not affected by the endogenous RM systems of hosts [45]. Thus, foreign DNAs can be introduced to *C. necator* via: (1) introducing DNA to a donor strain (the most commonly used one is *E. coli* S17-1) via chemical transformation or electroporation; (2) introducing DNA from S17-1 to *C. necator* via cell mating. This process requires two essential elements: a transfer gene (*tra*) and a mobilization site (*mob*) including the

Table 4 Commonly used *C. necator* strains for different applications

C. necator strain	Genome description	Application	Reference
H16PHB⁻4	Chemical mutates	Value-added products	[107]
H16Δ*phaCAB*	Precisely constructed to block PHB synthesis	Value-added products	[121]
H16Δ*phaC1*	Precisely constructed to block PHB synthesis	Value-added products	[83]
H16 G⁺1	UV mutates	Able to use glucose	[106]
C5	H16Δ*H16_A0006* Δ*H16_A0008-9*	Improved electroporation efficiency	[117]
Re2303	H16Δ(*H16_A0459-0464*, *H16_A1526-1531*); Mutant deficient in native β-oxidation	Fatty-acid derived fuels and chemicals	[124]
BC4	Mutant in *acrC1*, *Hfq*, *H16_A2360*, and *H16_B0214*	ROS-tolerant strain to be applied in MES	[10]
v6C6	Mutant in *H16_A0689*, *H16_A1373*, *H16_A2507*, and *H16_A3075*	Improved glycerol utilization	[120]

Pan *et al. Biotechnol Biofuels* (2021) 14:212

Page 10 of 20

origin of transfer (*oriT*) [45]. Commonly used plasmids in *C. necator* all have their own mobilization sequences. Gruber et al. showed that the mobilization efficiency of RP4 was about 10- and 50,000-fold higher than that of RSF1010 and pBBR1, respectively. The reason may lie in the donor strain S17-1, whose chromosome is integrated with the natural RP4 transfer sequences [126]. Mobilization sequences with high similarity to RP4 *mob* may perform interact better with RP4 transfer sequences and result in higher transformation efficiency [46]. Despite a high efficiency, conjugation requires a long experiment period and intensive labor with two rounds of cell culture. Therefore, a simple and time-saving method is preferred.

Electroporation

Electroporation is a more direct method, introducing foreign DNA into the recipient strain in a single step. However, the efficiency was as low as $10^2 \sim 10^3$ cfu/μg DNA depending on the plasmid size originally [127, 128]. Thus, two strategies have been employed to improve the transformation efficiency. Tee et al. systematically optimized the electroporation parameters, including transformation buffers, chemical treatment, electroporation voltage, cell concentration, and cell growth phase. The optimized electroporation protocol with cells grown to OD_{600} 0.6, a 15 min incubation in 50 mM $CaCl_2$, two cell washes with glycerol, resuspension in 0.2 M sucrose, and 2.3 kV electroporation, resulted in a transformation efficiency of $(3.86 \pm 0.29) \times 10^5$ cfu/μg DNA [125]. Xiong et al. found that the electroporation efficiency was limited by the endogenous RM systems and the disruption of the putative RM genes *H16_A0006* and *H16_A0008-9* in *C. necator* increased the electroporation efficiency more than 10^3 times [117]. These efforts have made electroporation feasible even for some large-size plasmids.

Chemico-physical transformation

Although common chemical transformation cannot get high enough transformation efficiency for *C. necator*, the combination with physical transformation (e.g., needle-like materials) makes it possible. Ren et al. tested the combination of five different chemicals (RbCl, lithium acetate, cesium chloride, dimethyl sulfoxide, and magnesium chloride) and four different nanomaterials (sepiolite, gold(III) chloride, multi-walled carbon nanotube, and chitosan) and found that the highest efficiency was obtained when cells were treated with gold(III) chloride and 0.1 M RbCl (3.49×10^4 CFU/μg of pBBR1MCS2). Although the transformation efficiency is slightly lower than electroporation (under optimal conditions), it is much simpler and no special equipment is required [129].

Synthetic biology applications of *C. necator* H16

With the development of genetic tools mentioned above, the use of *C. necator* as a biofuel-producing organism has become a hot spot [130]. Biofuel products that have been reported include alcohols (e.g., ethanol and isopropanol), fatty acids, ketones, alkanes, and terpenoids [131]. The general metabolic engineering strategy is to increase heterologous pathway activity and reduce the metabolic flux of competing pathways in *C. necator*. This section discusses the efforts that have been devoted to engineering *C. necator* with those above-mentioned genetic tools to synthesize biofuels and chemicals from CO_2. Notably, *C. necator* has been engineered to synthesize a wide variety of chemicals from organic sources, such as fructose and fatty acids, which is not the major focus of and not included in this review. The products synthesized in *C. necator* from CO_2 are listed in Table 5.

Alcohols
Isopropanol

Isopropanol (isopropyl alcohol or IPA) is mainly used as a solvent, but also as a feedstock to produce paints, cleaners, and cosmetic and chemical intermediates (such as esters and amines). The main driving force behind the development of fermentation production processes for pure IPA lies in the potential use as a precursor of isopropylene, which is one of the most important components of propylene in the chemical industry [132]. If the cost of IPA is low enough to serve as a biofuel molecule or a precursor of isopropylene, much larger markets can be expected [133]. Compared with the traditional chemical production method (e.g., by reducing acetone in the presence of excess hydrogen), microbial synthesis of IPA from renewable raw materials has great advantages, such as relatively mild conditions, no expensive catalysts required, and environmental friendly [134].

Cupriavidus necator can produce large amount of PHB under unfavorable growth conditions such as nutrient limitation to store excess carbon. Thus, *C. necator* is an excellent host to produce IPA, which shares the same biosynthesis precursors, acetoacetyl-CoA, with PHB (Fig. 3). A few genetic modifications are sufficient to rewire the metabolic fluxes of the precursor from PHB accumulation to IPA production. The deletion of competing pathways, *phaB* and *phaC*, and the introduction of the heterologous genes from *Clostridium species* (*adc* and *adh*) resulted in successful production of IPA. Then codon optimization and increasing copy numbers of the pathway genes were employed to increase the heterologous gene expression levels. Furthermore, by using an inducible promoter P*araBAD*, the engineered strain Re2133/pEG7c produced IPA with a titer up to 3.44 g/L
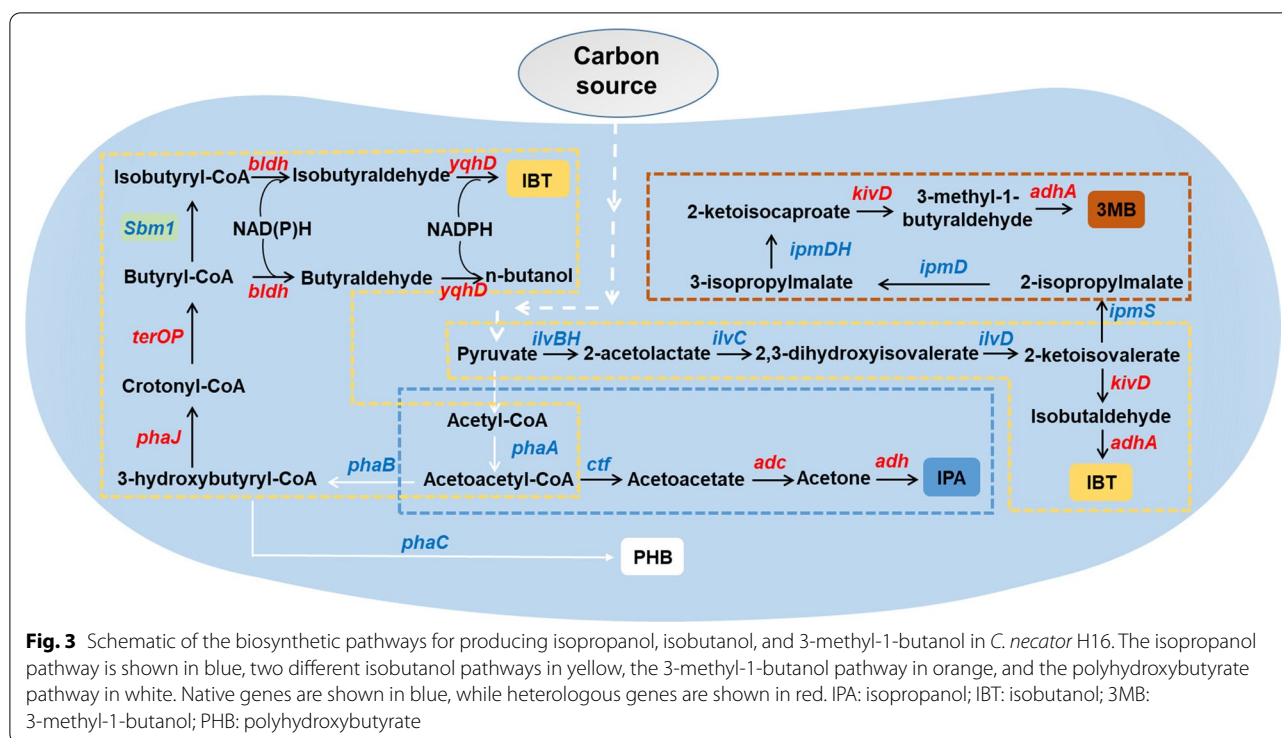
Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 11 of 20

Table 5 Products synthesized in *C. necator* from CO_2

Products	Vectors	Expression elements	Host	Fermentation conditions and titers (yields)	References
Acetoin	Plasmid (Tc^R, RSF1010, *par*)	P_{phb}-*alsSD*	H16Δ*acoABC*Δ*phaC1*Δ*phaC2*	Gas fermentation 0.32 mol acetoin/mol CO_2	[188]
2,3-Butanediol	Integration (*phaC1AB1*)	P_{araBAD}-*alsSD-sadh*	H16Δ*acoXABC* Δ*phaC1AB1*	Gas fermentation 32.0±0.1 g/L	[139]
Isopropanol	Integration (*phaC1*)	P_{araBAD}-*ctfAB-adc-sadh-phaA*	H16Δ*phaC1B1*	Gas fermentation 7.7±0.2 g/L	[139]
	Plasmid pEG7b (Kan^R, pBBR1)	P_{tac}-*thl-ctf-adc-adh*	H16Δ*phaCAB*Δ*phaB2* Δ*phaB3*	Gas fermentation 3.5 g/L	[138]
	Plasmid pEG12 (Kan^R, pBBR1)	P_{lac}-*thl-thl-ctf-adc-adh*	H16Δ*phaCAB*Δ*phaB2* Δ*phaB3*	MES 216 mg/L	[25]
	Plasmid pEG12 (Kan^R, pBBR1)	P_{lac}-*thl-thl-ctf-adc-adh*	H16Δ*phaCAB*Δ*phaB2* Δ*phaB3*	MES ~600 mg/L	[10]
n-Butanol	Plasmid pLH205 (Kan^R, pBHR1)	P_{CAT}-*phaJ-phaAB-terOP-bldh-yqhD*	H16	Formic acid 30 mg/L	[151]
Isobutanol and 3-methyl-1-butanol	Plasmid JL26 (Kan^R, pBBR1)	P_{lac}-*ilvBHCD-kivd*	DJ21Δ*phaCAB*Δ*ilvE* Δ*bkdAB*Δ*aceE*	MES ~220 mg/L	[10]
	Integration (*phaB2C2*) plasmid pYL22 (Kan^R, pBHR1)	int-P_{phaC1}-*alsS-ilvCD* plasmid-P_{CAT}-*kivd-yqhD*	H16Δ*phaCAB*	MES 140 mg/L	[26]
Fatty acids	Plasmids pCT (Kan^R, pBBR1); pFP (Cm^R, RP4)	pCT-P_{araBAD}-*acc-LTes* pFP-P_{araBAD}-*Fas-acpS*	H16Δ*phaC1*	Gas fermentation 60.64 mg/g CDW	[58]
Methyl ketones	Plasmid pJM20 (Kan^R, pBBR1)	P_{araBAD}- *'tesA-fadB-Mlut_11700-fadM*	Re2303Δ*phaCAB*	Gas fermentation 50~180 mg/L	[161]
Alka(e)ne	Plasmid pLC10 (Kan^R, pBBR1)	P_{araBAD}-*ado-aar*	H16Δ*phaCAB*	Gas fermentation 4.4 mg/L	[189]
α-Humulene	Plasmid pKR-hum (Tc^R, RSF1010, *par*)	P_{rhaBAD}-*zssl-erg20-hmgS-fni-hmgR-mvaK-mvaD-mvaK2*	H16PHB−4	MES 17 mg/g CDW	[19]
2-Hydroxy isobutyric acid	Plasmid pHCM (Kan^R, pBBR1)	P_{lac}-*meaB-hcmA-hcmB*	H16PHB−4	Gas fermentation 3.2 g/L	[190]
Trehalose	Plasmid pSEVA228 (Kan^R, RP4)	P_{xyls}-*setA*	H16	Gas fermentation 0.47 g/L	[191]
Sucrose	Plasmid pBADT (Kan^R, pBBR1)	P_{araBAD}-*SPS-SPP-scrY*	H16	Gas fermentation 180 mg/L	[192]
Lipochitooligosaccharides	Plasmid pBADT (Kan^R, pBBR1)	P_{araBAD}-*nodABC*	H16	Gas fermentation 1.4 mg/L	[192]
Lycopene	Plasmid (Kan^R, pBBR1)	P_{lac}-*CrtEBI2*	C5	MES 1.73 mg/L	[175]

DJ21is an isobutanol tolerant strain with constitutive alcohol dehydrogenase activity constructed by Jendrossek et al. [150]

from fructose as a sole carbon source [103]. Nevertheless, the potential product toxicity is another issue that should be addressed. As previously reported, heat shock proteins, alcohol dehydrogenases, and efflux pump proteins have been shown to increase ethanol tolerance in a broad range of bacteria such as *C. acetobutylicum* and *E. coli* [135, 136]. Thus, Marc et al. overexpressed GroES and GroEL (heat shock protein family) to increase the stability of ADC and ADH, leading to increased IPA production. Finally, *C. necator* Re2133/pEG23 strain was able to produce 9.8 g/L IPA when fructose was used as the sole carbon source [137]. To achieve IPA production from CO_2, Garrigues et al. designed a pressurized bioreactor to provide higher gas abundance and increase the gas transfer rate, with the IPA titer reaching as high as 3.5 g/L by gas fermentation [138]. Similarly, Bommareddy et al. established a continuous autotrophic fermentation system and obtained 7.7 g/L IPA [139]. Recently, an MES system was set up by coupling *C. necator* strain Re2133/pEG12 and water-splitting system, resulting in the production of 216 mg/L IPA [25]. Then, Liu et al. designed a novel electrode material to eliminate the generation

Pan *et al. Biotechnol Biofuels* (2021) 14:212

Page 12 of 20

Fig. 3 Schematic of the biosynthetic pathways for producing isopropanol, isobutanol, and 3-methyl-1-butanol in *C. necator* H16. The isopropanol pathway is shown in blue, two different isobutanol pathways in yellow, the 3-methyl-1-butanol pathway in orange, and the polyhydroxybutyrate pathway in white. Native genes are shown in blue, while heterologous genes are shown in red. IPA: isopropanol; IBT: isobutanol; 3MB: 3-methyl-1-butanol; PHB: polyhydroxybutyrate

of ROS and improved the titer of IPA to 600 mg/L with Co-P alloy cathode and CoPi anode [10]. Overall, great progress in IPA production from CO_2 in *C. necator* has been made in recent years, whose titer was far beyond those by cyanobacteria under photosynthetic conditions, i.e., ~150 mg/L [140–142].

Isobutanol and 3-methyl-1-butanol
Compared with isopropanol, higher alcohols (e.g., *n*-butanol, isobutanol, and 3-methyl-1-butanol) have higher energy density and lower vapor pressure, hygroscopicity, and water solubility [143]. C4 alcohols have been found to be compatible with the current fuel distribution infrastructure of most countries, and can be used as fuels to run vehicles without any gasoline blending. In addition, isobutanol (IBT) is an important precursor for isobutene, which is widely used in refineries, rubber, and special chemical industries [144, 145].

The branched-chain amino acids catabolic pathway was engineered to produce fusel alcohols [146]. In this so-called Ehrlich pathway, branched-chain amino acids were converted to branched-chain α-keto acids by amino transferase, which were subsequently decarboxylated into the corresponding aldehydes and further reduced to fusel alcohols [147] (Fig. 3). Many microorganisms such as *B. subtilis, Saccharomyces cerevisiae,* and *Lactococcus lactis* were reported to produce IBT and 3-methyl-1-butanol (3MB) via the Ehrlich pathway [147–149]. While for *C.*

necator, two additional enzymes (ketoisovalerate decarboxylase and alcohol dehydrogenase) should be introduced to produce IBT and 3MB [31]. A mutant strain of *C. necator* H16 with constitutive alcohol dehydrogenase activity and deficient PHB synthesis was chosen as the parent strain [150]. Subsequent overexpression of the heterologous *kivd* gene and the branched-chain amino acid biosynthesis pathway genes (*ilvBHCD*) resulted in the production of IBT and 3 MB. To further increase the production, other carbon sinks (i.e., valine-specific transaminase gene, a branched-chain keto acid dehydrogenase gene, and a pyruvate dehydrogenase gene) were deleted and the engineered strain produced 270 mg/L IBT and 40 mg/L 3 MB, respectively, when using fructose as the sole carbon source [121]. To enable IBT and 3 MB production from CO_2, Liu et al. inoculated this engineered strain in MES as well and finally the total titers of IBT and 3MB reached up to ~220 mg/L [10]. Similarly, an IBT-producing strain LH74D constructed by Li et al. was cultivated in MES. To minimize the cytotoxicity of ROS, a porous ceramic cup was used to shield the anode and thus provide more chances for ROS quenching. Such a system resulted in the production of over 140 mg/L (total of IBT and 3 MB) biofuels with electricity and CO_2 as the sole energy and carbon sources, respectively [26]. Notably, besides Ehrlich pathway, Black et al. designed a novel CoA-dependent pathway, constituted of chain elongation, rearrangement, and modification, for the synthesis

Pan *et al. Biotechnol Biofuels* (2021) 14:212

Page 13 of 20

of IBT. The endogenous isobutyryl-CoA mutase gene *Sbm1* was overexpressed to rearrange carbon flux from *n*-butanol to IBT and the engineered strain was able to produce 32 mg/L IBT with fructose as the sole carbon source [151] (Fig. 3). One of the factors limiting the ability of *C. necator* to produce IBT lies in low tolerance, i.e., lower than 0.5% (v/v). To address the product toxicity issues, Amanda and his colleagues constructed an IBT-tolerant strain by experimental evolution, which were able to grow in 2.5% (v/v) IBT and would be explored for IBT production in the near future [152]. Although the titer of IBT achieved in *C. necator* is far away from industrial application, the potential value of direct conversion of CO_2 to IBT is still appealing. The titer of IBT in cyanobacteria has been increased to as high as ~1 g/L [147, 153, 154], indicating room for further engineering of *C. necator*.

Fatty acids and derivatives

Fatty acids, a large category of important chemicals, have been the focus of metabolic engineering and can be further converted into valuable biofuels, e.g., fatty acid methyl esters (FAMEs) [155]. During fatty acid biosynthesis, acetyl-CoA is iteratively condensed on an acyl carrier protein (ACP) scaffold. However, fatty acids can also be consumed through the β-oxidation pathway (Fig. 4) [124, 156, 157]. Over 50 homologues of β-oxidation enzymes have been identified in the genome of *C. necator*, which is much more than other model organisms

such as *E. coli* [41]. Therefore, the synthesis of fatty acids and derivatives becomes a significant challenge in *C. necator*, which requires the block of β-oxidation pathway. Chen et al. overexpressed UcFatB2, which is a selective thioesterase for 12-carbon acyl-ACP substrates from the plant *Umbellularia californica*, in *C. necator* to produce laurate. The disruption of PHB synthesis and the acyl-CoA ligase gene *fadD3*, an entry point of fatty acids into β-oxidation, led to the production of total fatty acids increased up to 2.8-fold. Considering that laurate was still consumed in *C. necator*, three most highly upregulated acyl-CoA ligases were identified via RNA-Seq, the deletion of which resulted in the production of total fatty acids up to 62 mg/L [158]. Subsequently, Li et al. developed an autotrophic fermentation technique and obtained 60.64 mg/g CDW free fatty acids from CO_2, using *C. necator* with pCT-P$_{araBAD}$-*acc-LTes* and pFP-P$_{araBAD}$-*Fas-acpS* [58].

Alkanes are the predominant constituents of gasoline, diesel, and jet fuels [159]. Alkanes, which are synthesized from acyl-ACP, have been produced in several microorganisms including cyanobacteria, bacteria, yeast, and fungi [160]. To produce alkanes in *C. necator*, acyl-ACP reductase (*aar*) and aldehyde decarbonylase (*adc*) encoding genes were overexpressed with the genetic toolbox developed by Bi et al. and the engineered *C. necator* strain produced 6 mg/L of total hydrocarbons [47]. Similarly, Crepin et al. introduced acyl-ACP reductase (*aar*) and an aldehyde deformylating oxygenase (*ado*)
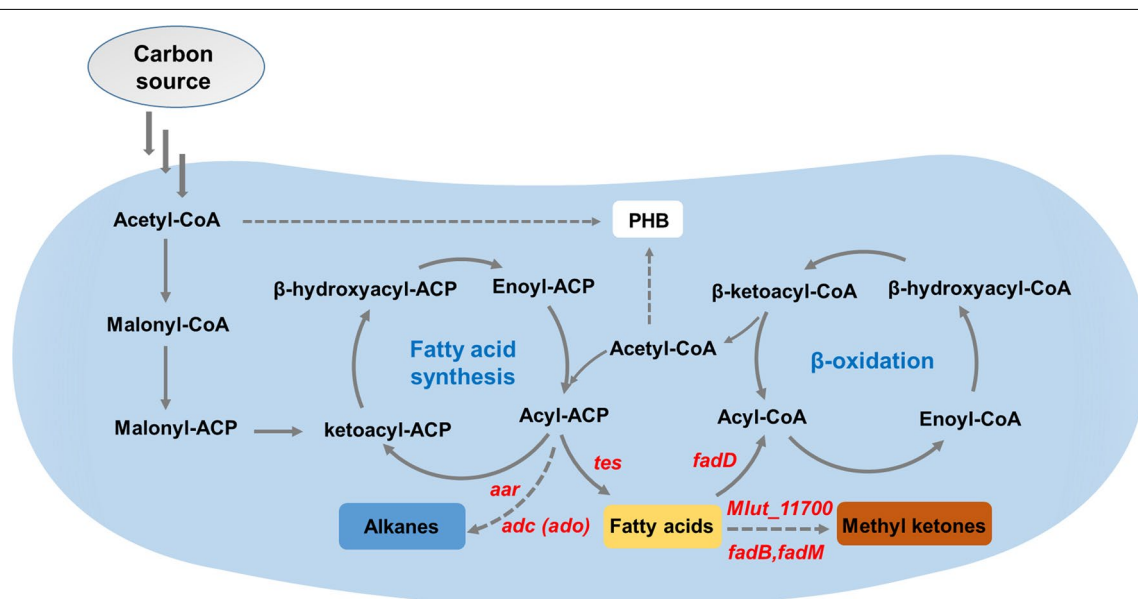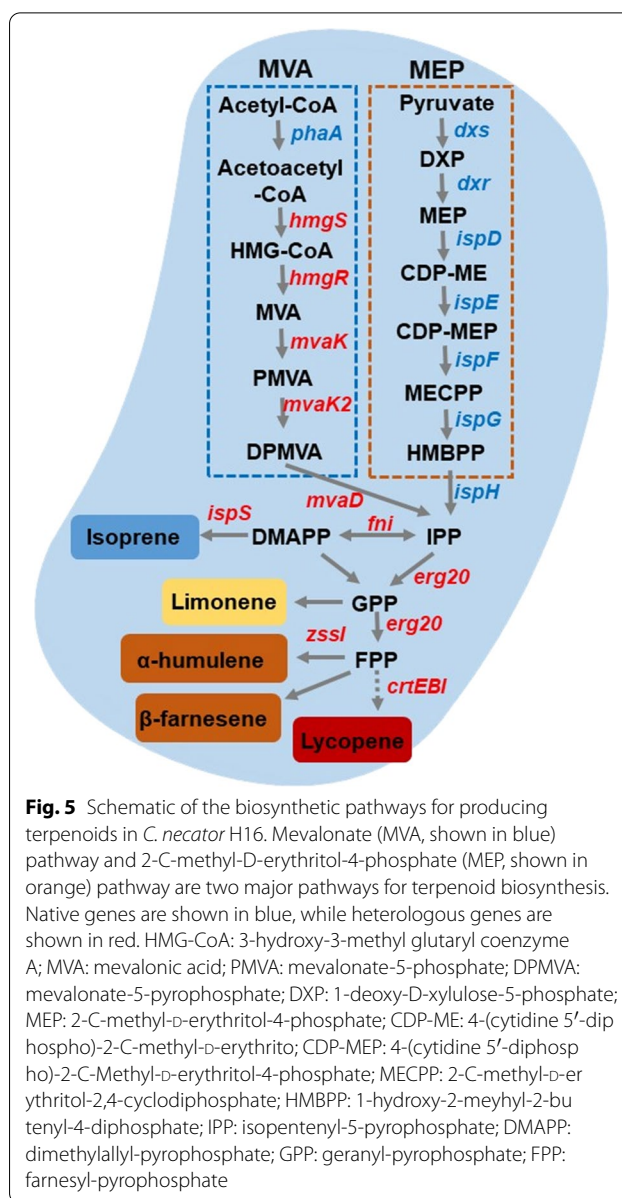

Fig. 4 Schematic of the biosynthetic pathways for producing fatty acids and derivatives in *C. necator* H16. Main related pathways include fatty acid biosynthesis, β-oxidation cycle, and PHB synthesis, with acetyl-CoA as a central building block. Heterologous genes to synthesize fatty acids, alkanes, and methyl ketones are shown in red. PHB: polyhydroxybutyrate

encoding genes to H16Δ*phaCAB*. Through codon, gene copy number, promoter, and RBS optimization, 435 mg/L of alkanes was produce from fructose and autotrophic alkane production was achieved. Although the autotrophic alkane production level was low (4.4 mg/L), it represented the first report to produce alka(e)nes from CO_2 [161]. Furthermore, Crepin et al. increased alka(e) nes production up to 1.48 g/L (from fructose) by the expression of endogenous and heterologous ferredoxin– ferredoxin reductase systems [162].

Medium-chain methyl ketones, commonly found in microorganisms, plants, insects, and mammalian cells [163], have a variety of applications, such as pheromones, natural insecticides, flavoring in food, and diesel fuel blending agents [164, 165]. *C. necator* is capable of producing methyl ketones by modifying fatty acids metabolism under heterotrophic and autotrophic conditions. As fatty acids are the precursor of methyl ketones, Müller et al. overexpressed a cytoplasmic version of the TesA thioesterase in Re2303Δ*phaCAB*, whose production of free fatty acids was more than 150-fold higher than that of the wild type. Subsequently, three heterologous genes (acyl-CoA oxidase gene *Mlut_11700* from *Micrococcus luteus* and *fadB* and *fadM* from *E. coli*) were overexpressed in *C. necator*, and the finally engineered *C. necator* strain produced methyl ketones up to 50~65 mg/L under heterotrophic conditions and 50~180 mg/L under chemolithoautotrophic growth conditions, respectively [161].

Isoprene and terpenes

Terpenoids are widespread in the nature. More than 22,000 terpenoids have been reported, representing the largest group of natural products [166]. Traditionally, terpenes are used as the key fragrance compounds in perfumes and medicines. Furthermore, terpenes can serve as substitutes for chlorinated solvents in applications such as cleaning of electronic components. Because the physicochemical properties of terpenes are similar to petroleum-based fuels, another potential application was the use as substitutes for petroleum fuels [167, 168].There are two main synthetic pathways of terpenes in nature: the classic mevalonate (MVA) pathway and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. The MVA pathway is widespread in eukaryotes, whereas the MEP pathway is prevalent in bacteria, e.g., *C. necator* H16. The MVA pathway converts acetyl-CoA to isopentenyl-5-pyrophosphate (IPP) and then an IPP isomerase maintains the balance between IPP and dimethylallyl-pyrophosphate (DMAPP). The MEP pathway consists of seven steps to convert glyceraldehyde-3-phosphate and pyruvate to IPP and DMAPP, which are building blocks for longer chain precursors of terpenes [169–171] (Fig. 5).

Fig. 5 Schematic of the biosynthetic pathways for producing terpenoids in *C. necator* H16. Mevalonate (MVA, shown in blue) pathway and 2-C-methyl-D-erythritol-4-phosphate (MEP, shown in orange) pathway are two major pathways for terpenoid biosynthesis. Native genes are shown in blue, while heterologous genes are shown in red. HMG-CoA: 3-hydroxy-3-methyl glutaryl coenzyme A; MVA: mevalonic acid; PMVA: mevalonate-5-phosphate; DPMVA: mevalonate-5-pyrophosphate; DXP: 1-deoxy-D-xylulose-5-phosphate; MEP: 2-C-methyl-D-erythritol-4-phosphate; CDP-ME: 4-(cytidine 5′-dip hospho)-2-C-methyl-D-erythrito; CDP-MEP: 4-(cytidine 5′-diphosp ho)-2-C-Methyl-D-erythritol-4-phosphate; MECPP: 2-C-methyl-D-er ythritol-2,4-cyclodiphosphate; HMBPP: 1-hydroxy-2-meyhyl-2-bu tenyl-4-diphosphate; IPP: isopentenyl-5-pyrophosphate; DMAPP: dimethylallyl-pyrophosphate; GPP: geranyl-pyrophosphate; FPP: farnesyl-pyrophosphate

Isoprene (C5) is widely used in the synthetic rubber industry and fuel additives, whose precursor is DMAPP. Lee et al. introduced MVA genes and an isoprene synthase gene (*ispS*) to *C. necator* H16 and obtained 3.8 μg/L isoprene through codon and promoter optimization [102]. Monoterpenoids (C10), such as limonene, have strong fragrance and biological activity, and are important raw materials in pharmaceutical, food, and cosmetic industries. Jannson et al. achieved the production of limonene by chemolithoautotrophic culture of engineered *C. necator* [172]. Sesquiterpenes (C15), the largest subgroup of terpenoids, has a wide application in industry, such as β-farnesene, a precursor for a jet fuel additive,

and α-humulene, a potential drug to treat cancer. Milker et al. obtained 26.3 ± 1.3 μM β-farnesene [173] and 2 g/L α-humulene [174] in a fed-batch mode on fructose as carbon source by expressing β-farnesene synthase and α-humulene synthase, respectively. Both studies showed that additional MVA expression contributed little to increase the titer of terpenoids, which might be resulted from the poor expression of hydroxymethylglutaryl-CoA reductase (*hmgR*). Furthermore, to produce terpenoids from CO_2 directly, Krieg et al. inoculated H16PHB-4/pKR-hum in MES to produce α-humulene, which is the first report on chemolithoautotrophic production of a terpene. The titer of α-humulene reached 10 mg/g cell dry weight (CDW) under heterotrophic conditions and 17 mg/g CDW under chemolithoautotrophic conditions [19]. Recently, Wu et al. used the CO_2 abundant real exhaust gas as the feedstock and achieved the production of 1.73 mg/L lycopene (C40), representing the most complex nonnative molecules in MES [175]. However, different with the production of diversified terpenes from CO_2 in cyanobacteria [176, 177], the terpenes spectrum of *C. necator* is still rather limited, indicating a need for more extensive studies.

Conclusions

This review summarizes genetic tools for *C. necator* from four perspectives: genetic engineering vectors, heterologous gene expression elements, platform strain and genome engineering, and transformation methods. Although many efforts have been devoted to expanding the toolkit, there remains an urgent need for more advanced methods, especially the CRISPR-based genome editing and gene regulation tools, e.g., CRISPRi [178] and base editor [179], to facilitate more complex metabolic engineering applications. Besides, computational simulation tools for *C. necator* are rather limited nowadays [180, 181]. To guide metabolic engineering, more in silico design tools, such as flux balance analysis (FBA) and elementary mode analysis (EMA), should be developed.

In addition, this review summarizes the value-added products converted from CO_2 in *C. necator*, including alcohols, fatty acids, and terpenoids. Although CO_2 valorization is mainly achieved via gas fermentation (H_2, O_2, and CO_2), MES is a more promising method. Nevertheless, many challenges remain for wide applications of MES: (1) to improve the efficiency of CO_2 fixation via metabolic engineering of CCB cycle or introducing new yet more efficient pathways; (2) to clarify and enhance the energy transfer process from the electrode to *C. necator*; (3) to diversify the product spectrum and increase the production yield; and (4) to better couple *C. necator* with the inorganic system.

In addition to CO_2 conversion, *C. necator* has been expanded for applications in new areas. For example, *C. necator* was used for artificial selection and directed evolution studies of RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase, the rate-limiting enzyme for CO_2 fixation) [182], the absorption and quantification of rare earth elements [183, 184], and hybrid photosynthesis [185–187]. With the development of more genetic engineering tools, the applications of *C. necator* are yet to be explored.

In summary, great progress has been made on genetic toolkit and synthetic biology applications of *C. necator*. Nevertheless, due to our limited knowledge of such a non-model microorganism, more efforts should be devoted to making *C. necator* as efficient cell factories for the conversion of CO_2 to value-added products.

Abbreviations
MES: Microbial electrosynthesis; ED: Entner–Doudoroff; CBB: Calvin–Benson–Basham; PHA: Polyhydroxyalkanoate; ORI: Origin of replication; MM: Minimal medium; PHB: Polyhydroxybutyrate; UTR: Untranslated region; CAP: Catabolite activator protein; IPTG: Isopropyl β-ᴅ-1-thiogalactopyranoside; MBH: Membrane-bound [NiFe]-hydrogenase; HR: Homologous recombination; sgRNA: Single guide RNA; DSB: Double-strand break; NHEJ: Non-homologous recombination end joining; ROS: Reactive oxygen species; NMG: 1-Nitroso-3-nitro-l-methylguanidine; RM: Restriction modification; IPA: Isopropanol; IBT: Isobutanol; 3MB: 3-Methyl-1-butanol; FAMEs: Fatty acid methyl esters; ACP: Acyl carrier protein; MVA: Mevalonate; MEP: 2-C-methyl-ᴅ-erythritol-4-phosphate; IPP: Isopentenyl-5-pyrophosphate; DMAPP: Dimethylallyl-pyrophosphate; FPP: Farnesyl pyrophosphate; CDW: Cell dry weight; RuBisCO: Ribulose 1,5-bisphosphate carboxylase/oxygenase; FBA: Flux balance analysis; EMA: Elementary mode analysis.

Authors' contributions
HP and JL conceived the review idea. HP and JW drafted the manuscript. All authors read and approved the final manuscript.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 16 of 20

Author details
[1]Key Laboratory of Biomass Chemical Engineering of Ministry of Education, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China. [2]Hangzhou Global Scientific and Technological Innovation Center, Zhejiang University, Hangzhou 310027, China.

References
1. Gernaat DE, de Boer H-S, Daioglou V, Yalew SG, Müller C, van Vuuren DP. Climate change impacts on renewable energy supply. Nat Clim Change. 2021;11(2):119–25.
2. Abanades JC, Rubin ES, Mazzotti M, Herzog HJ. On the climate change mitigation potential of CO_2 conversion to fuels. Energy Environ Sci. 2017;10(12):2491–9.
3. Tomei J, Helliwell R. Food versus fuel? Going beyond biofuels. Land Use Policy. 2016;56:320–6.
4. Curran LMLK, Sale KL, Simmons BA. Review of advances in the development of laccases for the valorization of lignin to enable the production of lignocellulosic biofuels and bioproducts. Biotechnol Adv. 2021. https://doi.org/10.1016/j.biotechadv.2021.107809.
5. Liu Z, Wang K, Chen Y, Tan T, Nielsen J. Third-generation biorefineries as the means to produce fuels and chemicals from CO_2. Nat Catal. 2020;3(3):274–88.
6. Kibria MG, Edwards JP, Gabardo CM, Dinh CT, Seifitokaldani A, Sinton D, et al. Electrochemical CO_2 reduction into chemical feedstocks: from mechanistic electrocatalysis models to system design. Adv Mater. 2019;31(31):e1807166.
7. Ross MB, De Luna P, Li Y, Dinh C-T, Kim D, Yang P, et al. Designing materials for electrochemical carbon dioxide recycling. Nat Catal. 2019;2(8):648–58.
8. Vijay D, Akhtar MK, Hess WR. Genetic and metabolic advances in the engineering of cyanobacteria. Curr Opin Biotechnol. 2019;59:150–6.
9. Dogutan DK, Nocera DG. Artificial photosynthesis at efficiencies greatly exceeding that of natural photosynthesis. Acc Chem Res. 2019;52(11):3143–8.
10. Liu C, Colón BC, Ziesack M, Silver PA, Nocera DG. Water splitting-biosynthetic system with CO_2 reduction efficiencies exceeding photosynthesis. Science. 2016;352(6290):1210–3.
11. Li H, Liao JC. Biological conversion of carbon dioxide to photosynthetic fuels and electrofuels. Energy Environ Sci. 2013;6(10):2892–9.
12. Rabaey K, Rozendal RA. Microbial electrosynthesis-revisiting the electrical route for microbial production. Nat Rev Microbiol. 2010;8(10):706–16.
13. Schlegel HG, Gottschalk G, Von Bartha R. Formation and utilization of poly-β-hydroxybutyric acid by *Knallgas* bacteria (*Hydrogenomonas*). Nature. 1961;191(4787):463–5.
14. Bowien B, Schlegel HG. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. Annu Rev Microbiol. 1981;35(1):405–52.
15. Vandamme P. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. Int J Syst Evol Microbiol. 2004;54(6):2285–9.
16. Walde E. Studies on growth and synthesis of stored substance by *Hydrogenomonas*. Arch Mikrobiol. 1962;43:109–37.
17. Panich J, Fong B, Singer SW. Metabolic engineering of *Cupriavidus necator* H16 for sustainable biofuels from CO_2. Trends Biotechnol. 2021;39(4):412–24.
18. Stockl M, Harms S, Dinges I, Dimitrova S, Holtmann D. From CO_2 to bioplastic-Coupling the electrochemical CO_2 reduction with a microbial product generation by drop-in electrolysis. Chemsuschem. 2020;13(16):4086–93.
19. Krieg T, Sydow A, Faust S, Huth I, Holtmann D. CO_2 to terpenes: autotrophic and electroautotrophic alpha-humulene production with *Cupriavidus necator*. Angew Chem Int Ed Engl. 2018;57(7):1879–82.
20. Chen X, Cao Y, Li F, Tian Y, Song H. Enzyme-assisted microbial electrosynthesis of poly(3-hydroxybutyrate) via CO_2 bioreduction by engineered *Ralstonia eutropha*. ACS Catal. 2018;8(5):4429–37.
21. Bause S, Decker M, Neubauer P, Vonau W. Optimization of the chemolithoautotrophic biofilm growth of *Cupriavidus necator* by means of electrochemical hydrogen synthesis. Chem Pap. 2018;72(5):1205–11.
22. Al Rowaihi IS, Paillier A, Rasul S, Karan R, Grotzinger SW, Takanabe K, et al. Poly(3-hydroxybutyrate) production in an integrated electromicrobial setup: Investigation under stress-inducing conditions. PLoS ONE. 2018;13(4):e0196079.
23. Sydow A, Krieg T, Ulber R, Holtmann D. Growth medium and electrolyte-How to combine the different requirements on the reaction solution in bioelectrochemical systems using *Cupriavidus necator*. Eng Life Sci. 2017;17(7):781–91.
24. Liu C, Nangle SN, Colon BC, Silver PA, Nocera DG. (13)C-Labeling the carbon-fixation pathway of a highly efficient artificial photosynthetic system. Faraday Discuss. 2017;198:529–37.
25. Torella JP, Gagliardi CJ, Chen JS, Bediako DK, Colon B, Way JC, et al. Efficient solar-to-fuels production from a hybrid microbial-water-splitting catalyst system. Proc Natl Acad Sci USA. 2015;112(8):2337–42.
26. Li H, Opgenorth PH, Wernick DG, Rogers S, Wu TY, Higashide W, et al. Integrated electromicrobial conversion of CO_2 to higher alcohols. Science. 2012;335(6076):1596.
27. Lu J, Brigham CJ, Li S, Sinskey AJ. *Ralstonia eutropha* H16 as a platform for the production of biofuels, biodegradable plastics, and fine chemicals from diverse carbon resources. Biotechnology for Biofuel Production and Optimization: Elsevier; 2016. p. 325-51
28. Cramm R. Genomic view of energy metabolism in *Ralstonia eutropha* H16. J Mol Microbiol Biotechnol. 2009;16(1–2):38–52.
29. Lenz O, Lauterbach L, Frielingsdorf S. O_2-tolerant [NiFe]-hydrogenases of *Ralstonia eutropha* H16: physiology, molecular biology, purification, and biochemical analysis. Methods Enzymol. 2018;613:117–51.
30. Lenz O, Lauterbach L, Frielingsdorf S, Friedrich B. 4 Oxygen-tolerant hydrogenases and their biotechnological potential. Biohydrogen: De Gruyter; 2015. p. 61-96.
31. Brigham CJ, Zhila N, Shishatskaya E, Volova TG, Sinskey AJ. Manipulation of *Ralstonia eutropha* carbon storage pathways to produce useful biobased products. In: Wang X, Chen J, Quinn P, editors. Reprogramming microbial metabolic pathways. Dordrecht: Springer; 2012. p. 343–66.
32. Reinecke F, Steinbuchel A. *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. J Mol Microbiol Biotechnol. 2009;16(1–2):91–108.
33. Volodina E, Raberg M, Steinbuchel A. Engineering the heterotrophic carbon sources utilization range of *Ralstonia eutropha* H16 for applications in biotechnology. Crit Rev Biotechnol. 2016;36(6):978–91.
34. Raberg M, Volodina E, Lin K, Steinbuchel A. *Ralstonia eutropha* H16 in progress: applications beside PHAs and establishment as production platform by advanced genetic tools. Crit Rev Biotechnol. 2018;38(4):494–510.
35. Little GT, Ehsaan M, Arenas-Lopez C, Jawed K, Winzer K, Kovacs K, et al. Complete genome sequence of *Cupriavidus necator* H16 (DSM 428). Microbiol Resour Announc. 2019;8(37):e00814.
36. Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, et al. Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat Biotechnol. 2006;24(10):1257–62.
37. Schwartz E, Henne A, Cramm R, Eitinger T, Friedrich B, Gottschalk G. Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H_2-based lithoautotrophy and anaerobiosis. J Mol Biol. 2003;332(2):369–83.
38. Alagesan S, Minton NP, Malys N. (13)C-assisted metabolic flux analysis to investigate heterotrophic and mixotrophic metabolism in *Cupriavidus necator* H16. Metabolomics. 2018;14(1):9.
39. Jugder BE, Chen Z, Ping DT, Lebhar H, Welch J, Marquis CP. An analysis of the changes in soluble hydrogenase and global gene expression in *Cupriavidus necator* (*Ralstonia eutropha*) H16 grown in heterotrophic diauxic batch culture. Microb Cell Fact. 2015;14:42.
40. Kohlmann Y, Pohlmann A, Schwartz E, Zuhlke D, Otto A, Albrecht D, et al. Coping with anoxia: a comprehensive proteomic and transcriptomic survey of denitrification. J Proteome Res. 2014;13(10):4325–38.
41. Shimizu R, Chou K, Orita I, Suzuki Y, Nakamura S, Fukui T. Detection of phase-dependent transcriptomic changes and Rubisco-mediated CO_2 fixation into poly (3-hydroxybutyrate) under heterotrophic condition in

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 17 of 20

Ralstonia eutropha H16 based on RNA-seq and gene deletion analyses. BMC Microbiol. 2013;13(1):169.

42. Schwartz E, Voigt B, Zuhlke D, Pohlmann A, Lenz O, Albrecht D, et al. A proteomic view of the facultatively chemolithoautotrophic lifestyle of *Ralstonia eutropha* H16. Proteomics. 2009;9(22):5132–42.

43. Peplinski K, Ehrenreich A, Doring C, Bomeke M, Reinecke F, Hutmacher C, et al. Genome-wide transcriptome analyses of the "Knallgas" bacterium *Ralstonia eutropha* H16 with regard to polyhydroxyalkanoate metabolism. Microbiology. 2010;156(Pt 7):2136–52.

44. Sharma PK, Fu J, Spicer V, Krokhin OV, Cicek N, Sparling R, et al. Global changes in the proteome of *Cupriavidus necator* H16 during poly-(3-hydroxybutyrate) synthesis from various biodiesel by-product substrates. AMB Express. 2016;6(1):1–6.

45. Gruber S, Schwab H, Koefinger P. Versatile plasmid-based expression systems for Gram-negative bacteria–General essentials exemplified with the bacterium *Ralstonia eutropha* H16. N Biotechnol. 2015;32(6):552–8.

46. Gruber S, Hagen J, Schwab H, Koefinger P. Versatile and stable vectors for efficient gene expression in *Ralstonia eutropha* H16. J Biotechnol. 2014;186:74–82.

47. Bi C, Su P, Müller J, Yeh Y-C, Chhabra SR, Beller HR, et al. Development of a broad-host synthetic biology toolbox for *Ralstonia eutropha* and its application to engineering hydrocarbon biofuel production. Microb Cell Fact. 2013;12(1):107.

48. Sato S, Fujiki T, Matsumoto K. Construction of a stable plasmid vector for industrial production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by a recombinant *Cupriavidus necator* H16 strain. J Biosci Bioeng. 2013;116(6):677–81.

49. Lutte S, Pohlmann A, Zaychikov E, Schwartz E, Becher JR, Heumann H, et al. Autotrophic production of stable-isotope-labeled arginine in *Ralstonia eutropha* strain H16. Appl Environ Microbiol. 2012;78(22):7884–90.

50. Fleige C, Kroll J, Steinbuchel A. Establishment of an alternative phosphoketolase-dependent pathway for fructose catabolism in *Ralstonia eutropha* H16. Appl Microbiol Biotechnol. 2011;91(3):769–76.

51. Budde CF, Riedel SL, Willis LB, Rha C, Sinskey AJ. Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. Appl Environ Microbiol. 2011;77(9):2847–54.

52. Voss I, Steinbuchel A. Application of a KDPG-aldolase gene-dependent addiction system for enhanced production of cyanophycin in *Ralstonia eutropha* strain H16. Metab Eng. 2006;8(1):66–78.

53. Srinivasan S, Barnard GC, Gerngross TU. A novel high-cell-density protein expression system based on *Ralstonia eutropha*. Appl Environ Microbiol. 2002;68(12):5925–32.

54. Srinivasan S, Barnard GC, Gerngross TU. Production of recombinant proteins using multiple-copy gene integration in high-cell-density fermentations of *Ralstonia eutropha*. Biotechnol Bioeng. 2003;84(1):114–20.

55. Tsuge T, Saito Y, Kikkawa Y, Hiraishi T, Doi Y. Biosynthesis and compositional regulation of poly[(3-hydroxybutyrate)-co-(3-hydroxyhexanoate)] in recombinant *Ralstonia eutropha* expressing mutated polyhydroxyalkanoate synthase genes. Macromol Biosci. 2004;4(3):238–42.

56. Lenz O, Strack A, Tran-Betcke A, Friedrich B. A hydrogen-sensing system in transcriptional regulation of hydrogenase gene expression in *Alcaligenes* species. J Bacteriol. 1997;179(5):1655–63.

57. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM II, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 1995;166(1):175–6.

58. Li Z, Xiong B, Liu L, Li S, Xin X, Li Z, et al. Development of an autotrophic fermentation technique for the production of fatty acids using an engineered *Ralstonia eutropha* cell factory. J Ind Microbiol Biotechnol. 2019;46(6):783–90.

59. Claassens NJ, Bordanaba-Florit G, Cotton CAR, De Maria A, Finger-Bou M, Friedeheim L, et al. Replacing the Calvin cycle with the reductive glycine pathway in *Cupriavidus necator*. Metab Eng. 2020;62:30–41.

60. Durland RH, Toukdarian A, Fang F, Helinski D. Mutations in the *trfA* replication gene of the broad-host-range plasmid RK2 result in elevated plasmid copy numbers. J Bacteriol. 1990;172(7):3859–67.

61. Sydow A, Pannek A, Krieg T, Huth I, Guillouet SE, Holtmann D. Expanding the genetic tool box for *Cupriavidus necator* by a stabilized L-rhamnose inducible plasmid system. J Biotechnol. 2017;263:1–10.

62. Balbás P. Understanding the art of producing protein and nonprotein molecules in *Escherichia coli*. Mol Biotechnol. 2001;19(3):251–67.

63. Yamaguchi Y, Park J-H, Inouye M. Toxin-antitoxin systems in bacteria and archaea. Annu Rev Genet. 2011;45:61–79.

64. Gerlitz M, Hrabak O, Schwab H. Partitioning of broad-host-range plasmid RP4 is a complex system involving site-specific recombination. J Bacteriol. 1990;172(11):6194–203.

65. Gruber S, Schwendenwein D, Magomedova Z, Thaler E, Hagen J, Schwab H, et al. Design of inducible expression vectors for improved protein production in *Ralstonia eutropha* H16 derived host strains. J Biotechnol. 2016;235:92–9.

66. Budde CF, Mahan AE, Lu J, Rha C, Sinskey AJ. Roles of multiple acetoacetyl coenzyme A reductases in polyhydroxybutyrate biosynthesis in *Ralstonia eutropha* H16. J Bacteriol. 2010;192(20):5319–28.

67. Pohlmann A, Cramm R, Schmelz K, Friedrich B. A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. Mol Microbiol. 2000;38(3):626–38.

68. Lenz O, Gleiche A, Strack A, Friedrich B. Requirements for heterologous production of a complex metalloenzyme: the membrane-bound [NiFe] hydrogenase. J Bacteriol. 2005;187(18):6590–5.

69. Delamarre SC, Batt CA. Comparative study of promoters for the production of polyhydroxyalkanoates in recombinant strains of *Wautersia eutropha*. Appl Microbiol Biotechnol. 2006;71(5):668–79.

70. Priefert H, Steinbüchel A. Identification and molecular characterization of the acetyl coenzyme A synthetase gene (*acoE*) of *Alcaligenes eutrophus*. J Bacteriol. 1992;174(20):6590–9.

71. Li H, Liao JC. A synthetic anhydrotetracycline-controllable gene expression system in *Ralstonia eutropha* H16. ACS Synth Biol. 2015;4(2):101–6.

72. Fukui T, Ohsawa K, Mifune J, Orita I, Nakamura S. Evaluation of promoters for gene expression in polyhydroxyalkanoate-producing *Cupriavidus necator* H16. Appl Microbiol Biotechnol. 2011;89(5):1527–36.

73. Arikawa H, Matsumoto K. Evaluation of gene expression cassettes and production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) with a fine modulated monomer composition by using it in *Cupriavidus necator*. Microb Cell Fact. 2016;15(1):184.

74. Alagesan S, Hanko EKR, Malys N, Ehsaan M, Winzer K, Minton NP, et al. Functional genetic elements for controlling gene expression in *Cupriavidus necator* H16. Appl Environ Microbiol. 2018;84(19):e00878.

75. Johnson AO, Gonzalez-Villanueva M, Tee KL, Wong TS. An engineered constitutive promoter set with broad activity range for *Cupriavidus necator* H16. ACS Synth Biol. 2018;7(8):1918–28.

76. Fukui T, Abe H, Doi Y. Engineering of *Ralstonia eutropha* for production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) from fructose and solid-state properties of the copolymer. Biomacromol. 2002;3(3):618–24.

77. Fukui T, Suzuki M, Tsuge T, Nakamura S. Microbial synthesis of poly ((R)-3-hydroxybutyrate-co-3-hydroxypropionate) from unrelated carbon sources by engineered *Cupriavidus necator*. Biomacromol. 2009;10(4):700–6.

78. Hanko EKR, Minton NP, Malys N. Characterisation of a 3-hydroxypropionic acid-inducible system from *Pseudomonas putida* for orthogonal gene expression control in *Escherichia coli* and *Cupriavidus necator*. Sci Rep. 2017;7(1):1724.

79. Hanko EKR, Minton NP, Malys N. A transcription factor-based biosensor for detection of itaconic acid. ACS Synth Biol. 2018;7(5):1436–46.

80. Aboulnaga EA, Zou H, Selmer T, Xian M. Development of a plasmid-based, tunable, tolC-derived expression system for application in *Cupriavidus necator* H16. J Biotechnol. 2018;274:15–27.

81. Barnard GC, Henderson GE, Srinivasan S, Gerngross TU. High level recombinant protein expression in *Ralstonia eutropha* using T7 RNA polymerase based amplification. Protein Expr Purif. 2004;38(2):264–71.

82. Hu M, Xiong B, Li Z, Liu L, Li S, Zhang C, et al. A novel gene expression system for *Ralstonia eutropha* based on the T7 promoter. BMC Microbiol. 2020;20(1):121.

83. York GM, Junker BH, Stubbe J, Sinskey AJ. Accumulation of the PhaP phasin of *Ralstonia eutropha* is dependent on production of polyhydroxybutyrate in cells. J Bacteriol. 2001;183(14):4217–26.

84. Bowien B, Kusian B. Genetics and control of CO_2 assimilation in the chemoautotroph *Ralstonia eutropha*. Arch Microbiol. 2002;178(2):85–93.

85. Dangel AW, Tabita FR. Amino acid substitutions in the transcriptional regulator CbbR lead to constitutively active CbbR proteins that elevate

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 18 of 20

expression of the cbb CO$_2$ fixation operons in *Ralstonia eutropha* (*Cupriavidus necator*) and identify regions of CbbR necessary for gene activation. Microbiology. 2015;161(9):1816–29.

86. Heinrich D, Raberg M, Steinbuchel A. Studies on the aerobic utilization of synthesis gas (syngas) by wild type and recombinant strains of *Ralstonia eutropha* H16. Microb Biotechnol. 2018;11(4):647–56.

87. Schwarze A, Kopczak MJ, Rogner M, Lenz O. Requirements for construction of a functional hybrid complex of photosystem I and [NiFe]-hydrogenase. App Environ Microbiol. 2010;76(8):2641–51.

88. Schwartz E, Buhrke T, Gerischer U, Friedrich B. Positive transcriptional feedback controls hydrogenase expression in *Alcaligenes eutrophus* H16. J Bacteriol. 1999;181(18):5684–92.

89. Schwartz E, Gerischer U, Friedrich B. Transcriptional regulation of *Alcaligenes eutrophus* hydrogenase genes. J Bacteriol. 1998;180(12):3197–204.

90. Jugder BE, Welch J, Braidy N, Marquis CP. Construction and use of a *Cupriavidus necator* H16 soluble hydrogenase promoter (PSH) fusion to gfp (green fluorescent protein). PeerJ. 2016;4:e2269.

91. Sinumvayo JP, Zhao C, Tuyishime P. Recent advances and future trends of riboswitches: attractive regulatory tools. World J Microbiol Biotechnol. 2018;34(11):171.

92. Serganov A, Nudler E. A decade of riboswitches. Cell. 2013;152(1–2):17–24.

93. Salis HM, Mirsky EA, Voigt CA. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol. 2009;27(10):946.

94. Mutalik VK, Guimaraes JC, Cambray G, Mai QA, Christoffersen MJ, Martin L, et al. Quantitative estimation of activity and quality for collections of functional genetic elements. Nat Methods. 2013;10(4):347–53.

95. Gai CS, Lu J, Brigham CJ, Bernardi AC, Sinskey AJ. Insights into bacterial CO$_2$ metabolism revealed by the characterization of four carbonic anhydrases in *Ralstonia eutropha* H16. AMB Express. 2014;4(1):2.

96. Tang R, Weng C, Peng X, Han Y. Metabolic engineering of *Cupriavidus necator* H16 for improved chemoautotrophic growth and PHB production under oxygen-limiting conditions. Metab Eng. 2020;61:11–23.

97. Peters V, Rehm BH. In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS Microbiol Lett. 2005;248(1):93–100.

98. Barnard GC, McCool JD, Wood DW, Gerngross TU. Integrated recombinant protein expression and purification platform based on *Ralstonia eutropha*. Appl Environ Microbiol. 2005;71(10):5735–42.

99. Yang TH, Kwon MA, Lee JY, Choi JE, Oh JY, Song JK. In situ immobilized lipase on the surface of intracellular polyhydroxybutyrate granules: preparation, characterization, and its promising use for the synthesis of fatty acid alkyl esters. Appl Biochem Biotechnol. 2015;177(7):1553–64.

100. Wong JX, Rehm BHA. Design of modular polyhydroxyalkanoate scaffolds for protein immobilization by directed ligation. Biomacromol. 2018;19(10):4098–112.

101. Huttanus HM, Feng X. Compartmentalized metabolic engineering for biochemical and biofuel production. Biotechnol J. 2017;12(6):1700052.

102. Lee HW, Park JH, Lee HS, Choi W, Seo SH, Anggraini ID, et al. Production of bio-based isoprene by the mevalonate pathway cassette in *Ralstonia eutropha*. J Microbiol Biotechnol. 2019;29(10):1656–64.

103. Grousseau E, Lu J, Gorret N, Guillouet SE, Sinskey AJ. Isopropanol production with engineered *Cupriavidus necator* as bioproduction platform. Appl Microbiol Biotechnol. 2014;98(9):4277–90.

104. Deaner M, Alper HS. Promoter and terminator discovery and engineering. In: Zhao H, Zeng A-P, editors. Synthetic biology-metabolic engineering. Cham: Springer; 2018. p. 21–44.

105. Orosz A, Boros I, Venetianer P. Analysis of the complex transcription termination region of the *Escherichia coli* rrn B gene. Eur J Biochem. 1991;201(3):653–9.

106. Schlegel H. Verwertung von Glucose durch eine Mutante von *Hydrogenomonas* H16. Biochim Z. 1965;341:249–59.

107. Schlegel H-G, Lafferty R, Krauss I. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch Mikrobiol. 1970;71(3):283–94.

108. Srivastava S, Urban M, Friedrich B. Mutagenesis of *Alcaligenes eutrophus* by insertion of the drug-resistance transposon Tn5. Arch Microbiol. 1982;131(3):203–7.

109. Peoples OP, Sinskey AJ. Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (phbC). J Biol Chem. 1989;264(26):15298–303.

110. Park JM, Jang Y-S, Kim TY, Lee SY. Development of a gene knockout system for *Ralstonia eutropha* H16 based on the broad-host-range vector expressing a mobile group II intron. FEMS Microbiol Lett. 2010;309(2):193–200.

111. Alexeyev MF. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. Biotechniques. 1999;26(5):824–8.

112. Lenz O, Schwartz E, Dernedde J, Eitinger M, Friedrich B. The *Alcaligenes eutrophus* H16 hoxX gene participates in hydrogenase regulation. J Bacteriol. 1994;176(14):4385–93.

113. Quandt J, Hynes MF. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene. 1993;127(1):15–21.

114. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene. 1994;145(1):69–73.

115. Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis. 2000;26(2):99–109.

116. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014;346(6213):1258096.

117. Xiong B, Li Z, Liu L, Zhao D, Zhang X, Bi C. Genome editing of *Ralstonia eutropha* using an electroporation-based CRISPR-Cas9 technique. Biotechnol Biofuels. 2018;11(1):1–9.

118. Feng X, Zhao D, Zhang X, Ding X, Bi C. CRISPR/Cas9 assisted multiplex genome editing technique in *Escherichia coli*. Biotechnol J. 2018;13(9):1700604.

119. Banno S, Nishida K, Arazoe T, Mitsunobu H, Kondo A. Deaminase-mediated multiplex genome editing in *Escherichia coli*. Nat Microbiol. 2018;3(4):423–9.

120. Gonzalez-Villanueva M, Galaiya H, Staniland P, Staniland J, Savill I, Wong TS, et al. Adaptive laboratory evolution of *Cupriavidus necator* H16 for carbon co-utilization with glycerol. Int J Mol Sci. 2019;20(22):5735.

121. Lu J, Brigham CJ, Gai CS, Sinskey AJ. Studies on the production of branched-chain alcohols in engineered *Ralstonia eutropha*. Appl Microbiol Biotechnol. 2012;96(1):283–97.

122. Raberg M, Peplinski K, Heiss S, Ehrenreich A, Voigt B, Döring C, et al. Proteomic and transcriptomic elucidation of the mutant *Ralstonia eutropha* G+1 with regard to glucose utilization. Appl Environ Microbiol. 2011;77(6):2058–70.

123. Raberg M, Kaddor C, Kusian B, Stahlhut G, Budinova R, Kolev N, et al. Impact of each individual component of the mutated PTS (Nag) on glucose uptake and phosphorylation in *Ralstonia eutropha* G(+)1. Appl Microbiol Biotechnol. 2012;95(3):735–44.

124. Brigham CJ, Budde CF, Holder JW, Zeng Q, Mahan AE, Rha C, et al. Elucidation of β-oxidation pathways in *Ralstonia eutropha* H16 by examination of global gene expression. J Bacteriol. 2010;192(20):5454–64.

125. Tee KL, Grinham J, Othusitse AM, González-Villanueva M, Johnson AO, Wong TS. An efficient transformation method for the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Biotechnol J. 2017;12(11):1700081.

126. Simon R, Priefer U, Pühler A. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technol. 1983;1(9):784–91.

127. Park H-C, Lim K-J, Park J-S, Lee Y-H, Huh T-L. High frequency transformation of *Alcaligenes eutrophus* producing poly-β-hydroxybutyric acid by electroporation. Biotechnol Tech. 1995;9(1):31–4.

128. Solaiman DKY, Swingle BM, Ashby RD. A new shuttle vector for gene expression in biopolymer-producing *Ralstonia eutropha*. J Microbiol Meth. 2010;82(2):120–3.

129. Ren J, Na D, Yoo SM. Optimization of chemico-physical transformation methods for various bacterial species using diverse chemical compounds and nanomaterials. J Biotechnol. 2018;288:55–60.

130. Chakravarty J, Brigham CJ. Solvent production by engineered *Ralstonia eutropha*: channeling carbon to biofuel. Appl Microbiol Biotechnol. 2018;102(12):5021–31.

131. Lee H-M, Jeon B-Y, Oh M-K. Microbial production of ethanol from acetate by engineered *Ralstonia eutropha*. Biotechnol Bioproc E. 2016;21:402–7.

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 19 of 20

132. Walther T, Francois JM. Microbial production of propanol. Biotechnol Adv. 2016;34(5):984–96.

133. Shen CR, Liao JC. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab Eng. 2008;10(6):312–20.

134. Choi KY, Wernick DG, Tat CA, Liao JC. Consolidated conversion of protein waste into biofuels and ammonia using *Bacillus subtilis*. Metab Eng. 2014;23:53–61.

135. Dunlop MJ. Engineering microbes for tolerance to next-generation biofuels. Biotechnol Biofuels. 2011;4(1):1–9.

136. Mukhopadhyay A. Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. Trends Microbiol. 2015;23(8):498–508.

137. Marc J, Grousseau E, Lombard E, Sinskey AJ, Gorret N, Guillouet SE. Over expression of GroESL in *Cupriavidus necator* for heterotrophic and autotrophic isopropanol production. Metab Eng. 2017;42:74–84.

138. Garrigues L, Maignien L, Lombard E, Singh J, Guillouet SE. Isopropanol production from carbon dioxide in *Cupriavidus necator* in a pressurized bioreactor. New Biotechnol. 2020;56:16–20.

139. Bommareddy RR, Wang Y, Pearcy N, Hayes M, Lester E, Minton NP, et al. A sustainable chemicals manufacturing paradigm using CO_2 and renewable H_2. iScience. 2020;23(6):101218.

140. Hirokawa Y, Dempo Y, Fukusaki E, Hanai T. Metabolic engineering for isopropanol production by an engineered cyanobacterium, *Synechococcus elongatus* PCC 7942, under photosynthetic conditions. J Biosci Bioeng. 2017;123(1):39–45.

141. Hirokawa Y, Suzuki I, Hanai T. Optimization of isopropanol production by engineered cyanobacteria with a synthetic metabolic pathway. J Biosci Bioeng. 2015;119(5):585–90.

142. Kusakabe T, Tatsuke T, Tsuruno K, Hirokawa Y, Atsumi S, Liao JC, et al. Engineering a synthetic pathway in cyanobacteria for isopropanol production directly from carbon dioxide and light. Metab Eng. 2013;20:101–8.

143. Atsumi S, Hanai T, Liao JC. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature. 2008;451(7174):86–9.

144. Gogerty DS, Bobik TA. Formation of isobutene from 3-hydroxy-3-methylbutyrate by diphosphomevalonate decarboxylase. Appl Environ Microbiol. 2010;76(24):8004–10.

145. Brigham CJ, Gai CS, Lu J, Speth DR, Worden RM, Sinskey AJ. Engineering *Ralstonia eutropha* for production of isobutanol from CO_2, H_2, and O_2. Advanced biofuels and bioproducts: Springer; 2013. p. 1065–90.

146. Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol. 2008;74(8):2259–66.

147. Atsumi S, Higashide W, Liao JC. Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. Nat Biotechnol. 2009;27(12):1177–80.

148. Yan Y, Liao JC. Engineering metabolic systems for production of advanced fuels. J Ind Microbiol Biotechnol. 2009;36(4):471–9.

149. Savrasova EA, Kivero AD, Shakulov RS, Stoynova NV. Use of the valine biosynthetic pathway to convert glucose into isobutanol. J Ind Microbiol Biotechnol. 2011;38(9):1287–94.

150. Jendrossek DI, Krüger N, Steinbüchel A. Characterization of alcohol dehydrogenase genes of derepressible wild-type *Alcaligenes eutrophus* H16 and constitutive mutants. J Bacteriol. 1990;172(9):4844–51.

151. Black WB, Zhang L, Kamoku C, Liao JC, Li H. Rearrangement of coenzyme A-acylated carbon chain enables synthesis of isobutanol via a novel pathway in *Ralstonia eutropha*. ACS Synth Biol. 2018;7(3):794–800.

152. Bernardi AC, Gai CS, Lu J, Sinskey AJ, Brigham CJ. Experimental evolution and gene knockout studies reveal AcrA-mediated isobutanol tolerance in *Ralstonia eutropha*. J Biosci Bioeng. 2016;122(1):64–9.

153. Wu XX, Li JW, Xing SF, Chen HT, Song C, Wang SG, et al. Establishment of a resource recycling strategy by optimizing isobutanol production in engineered cyanobacteria using high salinity stress. Biotechnol Biofuels. 2021;14(1):1–15.

154. Miao R, Xie H, Lindblad P. Enhancement of photosynthetic isobutanol production in engineered cells of *Synechocystis* PCC 6803. Biotechnol Biofuels. 2018;11:1–9.

155. Westbrook CK. Biofuels combustion. Annu Rev Phys Chem. 2013;64:201–19.

156. Riedel SL, Lu J, Stahl U, Brigham CJ. Lipid and fatty acid metabolism in *Ralstonia eutropha*: relevance for the biotechnological production of value-added products. Appl Microbiol Biotechnol. 2014;98(4):1469–83.

157. Insomphun C, Mifune J, Orita I, Numata K, Nakamura S, Fukui T. Modification of β-oxidation pathway in *Ralstonia eutropha* for production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from soybean oil. J Biosci Bioeng. 2014;117(2):184–90.

158. Chen JS, Colon B, Dusel B, Ziesack M, Way JC, Torella JP. Production of fatty acids in *Ralstonia eutropha* H16 by engineering beta-oxidation and carbon storage. PeerJ. 2015;3:e1468.

159. Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD. Microbial engineering for the production of advanced biofuels. Nature. 2012;488(7411):320–8.

160. Ladygina N, Dedyukhina EG, Vainshtein MB. A review on microbial synthesis of hydrocarbons. Process Biochem. 2006;41(5):1001–14.

161. Muller J, MacEachran D, Burd H, Sathitsuksanoh N, Bi C, Yeh YC, et al. Engineering of *Ralstonia eutropha* H16 for autotrophic and heterotrophic production of methyl ketones. Appl Environ Microbiol. 2013;79(14):4433–9.

162. Crépin L, Barthe M, Leray F, Guillouet SE. Alka(e)ne synthesis in *Cupriavidus necator* boosted by the expression of endogenous and heterologous ferredoxin–ferredoxin reductase systems. Biotechnol Bioeng. 2018;115(10):2576–84.

163. Beller HR, Lee TS, Katz L. Natural products as biofuels and bio-based chemicals: fatty acids and isoprenoids. Nat Prod Rep. 2015;32(10):1508–26.

164. Antonious GF, Dahlman DL, Hawkins LM. Insecticidal and acaricidal performance of methyl ketones in wild tomato leaves. Bull Environ Contam Toxicol. 2003;71(2):400–7.

165. Goh EB, Baidoo EE, Keasling JD, Beller HR. Engineering of bacterial methyl ketone synthesis for biofuels. Appl Environ Microbiol. 2012;78(1):70–80.

166. De Carvalho CC, da Fonseca MMR. Biotransformation of terpenes. Biotechnol Adv. 2006;24(2):134–42.

167. Kirby J, Keasling JD. Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu Rev Plant Biol. 2009;60:335–55.

168. Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS. Identification and microbial production of a terpene-based advanced biofuel. Nat Commun. 2011;2(1):1–8.

169. Cunningham FX Jr, Lafond TP, Gantt E. Evidence of a role for *LytB* in the nonmevalonate pathway of isoprenoid biosynthesis. J Bacteriol. 2000;182:5841–8.

170. Harada H, Yu F, Okamoto S, Kuzuyama T, Utsumi R, Misawa N. Efficient synthesis of functional isoprenoids from acetoacetate through metabolic pathway-engineered *Escherichia coli*. Appl Microbiol Biotechnol. 2009;81(5):915–25.

171. Sonntag F, Kroner C, Lubuta P, Peyraud R, Horst A, Buchhaupt M, et al. Engineering *Methylobacterium extorquens* for de novo synthesis of the sesquiterpenoid alpha-humulene from methanol. Metab Eng. 2015;32:82–94.

172. Jannson C, Carr CAM, Reed JS. Microorganisms for biosynthesis of limonene on gaseous substrates. Google Patents; 2016.

173. Milker S, Holtmann D. First time beta-farnesene production by the versatile bacterium *Cupriavidus necator*. Microb Cell Fact. 2021;20(1):1–7.

174. Milker S, Sydow A, Torres-Monroy I, Jach G, Faust F, Kranz L, et al. Gram-scale production of the sesquiterpene alpha-humulene with *Cupriavidus necator*. Biotechnol Bioeng. 2021;118(7):2694–702.

175. Wu H, Pan H, Li Z, Liu T, Liu F, Xiu S, et al. Efficient production of lycopene from CO_2 via microbial electrosynthesis. Chem Eng J. 2021. https://doi.org/10.1016/j.cej.2021.132943.

176. Lin PC, Pakrasi HB. Engineering cyanobacteria for production of terpenoids. Planta. 2019;249(1):145–54.

177. Ni J, Tao F, Xu P, Yang C. Engineering cyanobacteria for photosynthetic production of C3 platform chemicals and terpenoids from CO_2. In: Zhang W, Song X, editors. Synthetic biology of cyanobacteria. Singapore: Springer; 2018. p. 239–59.

178. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 2013;152(5):1173–83.

Pan *et al. Biotechnol Biofuels*   (2021) 14:212

Page 20 of 20

179. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 2016;533(7603):420–4.

180. Dalwadi MP, Garavaglia M, Webb JP, King JR, Minton NP. Applying asymptotic methods to synthetic biology: Modelling the reaction kinetics of the mevalonate pathway. J Theor Biol. 2018;439:39–49.

181. Unrean P, Tee KL, Wong TS. Metabolic pathway analysis for in silico design of efficient autotrophic production of advanced biofuels. Bioresour Bioprocess. 2019;6(1):1–11.

182. Satagopan S, Tabita FR. RubisCO selection using the vigorously aerobic and metabolically versatile bacterium *Ralstonia eutropha*. FEBS J. 2016;283(15):2869–80.

183. Adekanmbi EO, Giduthuri AT, Waymire S, Srivastava SK. Utilization of dielectrophoresis for the quantification of rare earth elements adsorbed on *Cupriavidus necator*. ACS Sustain Chem Eng. 2019;8(3):1353–61.

184. Giduthuri AT, Adekanmbi EO, Srivastava SK, Moberly JG. Dielectrophoretic ultra-high-frequency characterization and in silico sorting on uptake of rare earth elements by *Cupriavidus necator*. Electrophoresis. 2021;42(5):656–66.

185. Xu M, Tremblay P-L, Jiang L, Zhang T. Stimulating bioplastic production with light energy by coupling *Ralstonia eutropha* with the photocatalyst graphitic carbon nitride. Green Chem. 2019;21(9):2392–400.

186. Tremblay P-L, Xu M, Chen Y, Zhang T. Nonmetallic abiotic-biological hybrid photocatalyst for visible water splitting and carbon dioxide reduction. iScience. 2020;23(1):100784.

187. Xu M, Tremblay PL, Ding R, Xiao J, Wang J, Kang Y, et al. Photo-augmented PHB production from CO_2 or fructose by *Cupriavidus necator* and shape-optimized CdS nanorods. Sci Total Environ. 2021;753:142050.

188. Windhorst C, Gescher J. Efficient biochemical production of acetoin from carbon dioxide using *Cupriavidus necator* H16. Biotechnol Biofuels. 2019;12(1):1–11.

189. Crépin L, Lombard E, Guillouet SE. Metabolic engineering of *Cupriavidus necator* for heterotrophic and autotrophic alka(e)ne production. Metab Eng. 2016;37:92–101.

190. Przybylski D, Rohwerder T, Dilssner C, Maskow T, Harms H, Muller RH. Exploiting mixtures of H_2, CO_2, and O_2 for improved production of methacrylate precursor 2-hydroxyisobutyric acid by engineered *Cupriavidus necator* strains. Appl Microbiol Biotechnol. 2015;99(5):2131–45.

191. Lowe H, Beentjes M, Pfluger-Grau K, Kremling A. Trehalose production by *Cupriavidus necator* from CO_2 and hydrogen gas. Bioresour Technol. 2021;319:124–69.

192. Nangle SN, Ziesack M, Buckley S, Trivedi D, Loh DM, Nocera DG, et al. Valorization of CO_2 through lithoautotrophic production of sustainable chemicals in *Cupriavidus necator*. Metab Eng. 2020;62:207–20.

Publisher's Note