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Review

Prospects for engineering *Ralstonia eutropha* and *Zymomonas mobilis* for the autotrophic production of 2,3-butanediol from CO_2 and H_2

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ABSTRACT

The decarbonization of the chemical industry and a shift toward circular economies because of high global CO_2 emissions make CO_2 an attractive feedstock for manufacturing chemicals. Moreover, H_2 is a low-cost and carbon-free reductant because technologies such as solar-driven electrolysis and supercritical water (scH_2O) gasification enable sustainable production of molecular hydrogen (H_2). We review the recent advances in engineering *Ralstonia eutropha*, the representative species of "Knallgas" bacteria, for utilizing CO_2 and H_2 to autotrophically produce 2,3-butanediol (2,3-BDO). This assessment is focused on state-of-the-art approaches for splitting H_2 to supply energy in the form of ATP and NADH to power cellular reactions and employing the Calvin-Benson-Bassham cycle for CO_2 fixation. Major challenges and opportunities for application and future perspectives are discussed in the context of developing other promising CO_2 and H_2-utilizing microorganisms, exemplified by *Zymomonas mobilis*.

1. Introduction

In the past half-century, burning fossil fuels, deforestation, and other factors (such as urbanization and waste decomposing in landfills) contributed to global CO_2 emissions increasing to approximately 420 ppm. CO_2 accounts for about 77% (v/v) of the total greenhouse gas atmospheric sink [1], and its concentration has been increasing since industrialization. The rising concentration of CO_2 in the atmosphere drives adverse primary and secondary environmental changes, which include global warming, leading to rising sea levels, species migrations, and weather anomalies, and ocean acidification, resulting in the shrinking of coral reefs and stochastic alterations in aquatic biota. Thus, it is imperative to develop effective technologies for CO_2 capture, storage, sequestration [2], and for converting CO_2 to chemicals and cell mass by plants, algae, *cyanobacteria*, and various microorganisms. A detailed list of suitable CO_2-fixing and reducing microorganisms is described in a recent review [3]. Gas fermentation using these CO_2-fixing microorganisms presents promising opportunities for capturing carbon oxides from gaseous waste streams and recycling this carbon, thus enabling a circular, low-carbon future economy.

Ralstonia eutropha (also known as *Cupriavidus necator*) is a member of the Knallgas bacteria, a physiologic group of bacteria defined by their ability to grow autotrophically by gaining energy from H_2 and O_2 gas mixtures by using O_2 as an electron acceptor and H_2 as an electron donor [4]. *R. eutropha* is a well-studied model facultative bacterium that can use gluconic acid, fructose, and other organic carbon substrates for

Abbreviations: acoXABC, operon encoding genes for cleavage of acetoin; ADH, alcohol dehydrogenase; ALDC, acetolactate decarboxylase; ALS, 2-acetolactate synthase; ATP-PFK, ATP-dependent phosphofructokinase; BDH, butanediol dehydrogenase; BDO, butanediol; CA, carbonic anhydrase; CBB, Calvin-Benson-Bassham; CCM, CO_2 concentrating mechanism; CO_2, carbon dioxide; CydA, cytochrome bd-I ubiquinol oxidase subunit 1; CydB, cytochrome bd-I ubiquinol oxidase subunit 2; Cyt A, cyt ochrome A; DHAP, dihydroxyacetone phosphate; ED, Entner-Doudoroff; EDA, 2-keto-3-deoxy-phosphogluconate aldolase; EDD, 6-phosphogluconate dehydratase; EMP, Embden-Meyerhof-Parnas; ENO, enolase; FBA, fructose bisphosphate aldolase; G3P, glyceraldehyde 3-phosphate; GAP, glyceraldehyde 3-phosphate dehydrogenase; GLK, glucokinase; KC, apparent Km for CO_2; KDPG, 2-keto-3-deoxy-6-phosphogluconate; LDH, lactate dehydrogenase; MBH, membrane-bound hydrogenase; Ndh, NADH dehydrogenase; OAA, oxaloacetate; PAS, Per-Arnt-Sim domain; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PGI, phosphoglucose isomerase; PGL, phosphogluconolactonase; PGM, phosphoglyceromutase; PHA, poly-3-hydroxyalkanoates; phaCAB, the operon of phaC coding for PHA synthase, phaA for β-ketothiolase, and phaB for acetoacetyl-CoA reductase; PHB, polyhydroxybutyrate; PPi-PFK, PPi-dependent phosphofructokinase; PPP, pentose phosphate pathway; PRK, phosphoribulokinase; PYK, pyruvate kinase; Rubisco, ribulose1,5 bisphosphate carboxylase/oxygenase; RH, regulatory hydrogenase; scH_2O, supercritical water; SH, soluble hydrogenase; TCA, tricarboxylic acid cycle; TPI, triosephosphate isomerase; ZWF, glucose 6-phosphate dehydrogenase.

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heterogeneous growth and can also use CO_2 for autotrophic growth, as reviewed in a recent literature [3]. The ability of *R. eutropha* to utilize H_2 and CO_2 makes it an attractive model microorganism for the sustainable production of valuable chemicals under autotrophic conditions [5]. In recent years, *R. eutropha* has been successfully engineered, by blocking the PHB production, to produce a broad range of chemicals, including isoprenoids, terpenes [6], and BDO, which is the focus of this review. We also discuss the opportunities and gaps for carbon-negative BDO production from recycled carbon by engineering 2,3-BDO-producing *Zymomonas mobilis* strains to utilize CO_2 and H_2.

2. H_2 sources and the utilization of H_2 as a reducing power by *R. eutropha*

R. eutropha is a chemolithoautotrophic bacterium that can utilize H_2 as an electron donor, CO_2 as the sole carbon source, and O_2 as the electron acceptor for aerobic, autotrophic growth [5]. Molecular hydrogen (H_2) is a clean and renewable alternative fuel with an energy density per weight ratio far larger than gasoline or diesel. The combustion of H_2 with O_2 is environment-friendly as its only byproduct is a liquid water [7]. H_2 is widely used as the reducing agent of choice to catalyze chemical reactions in the chemical industry. In natural environments, the reducing power of H_2 has been exploited by a broad range of microorganisms as an energy source for their metabolism [7].

A few sustainable technologies have emerged for the renewable production of H_2 as a low-cost and carbon-free reductant. Such technologies include **(1)** solar-driven electrolysis [8], **(2)** supercritical water (scH_2O) gasification [9], and **(3)** biomass pyrolysis-gasification [10]. These technologies will make the reducing power of H_2 available for *in vivo* biosynthesis of chemicals and support the downstream scale-up.

R. eutropha can split H_2 to supply energy in the forms of ATP and NADH, which are subsequently used for cellular reactions. Hydrogenases are metalloenzymes that convert H_2 to $2H^+ + 2e^-$, with a redox potential (E_o') of −414 mV [11]. Notably, the hydrogenases of strain H16 are unique; they are tolerant to oxygen and able to sustain catalytic activity in the presence of O2, the final electron acceptor in *R. eutropha* [12]. In contrast, most other hydrogenases in other microorganisms are readily inactivated by O_2 [7].

In total, *R. eutropha* H16 expresses four catalytically active hydrogenases, as illustrated in Fig. 1 and is described below:

(1) The membrane-bound hydrogenase (MBH) is a [NiFe] hydrogenase comprising HoxG and HoxK. It reduces ubiquinone, delivers electrons from H_2 oxidation to cytochrome *a* (Cyt *a*) of the respiratory chain, and generates the proton motive force needed for ATP synthesis via ATPase [13,14].
(2) The soluble hydrogenase (SH) in *R. eutropha* is capable of oxidizing H_2 gas and using electrons to reduce NAD^+ to NADH. Importantly, via NADH oxidation, $NADP^+$ is reduced to NADPH, which drives metabolism [13,14], as illustrated in Fig. 1. SH is encoded by the operon of hoxFUYHWI, in which HoxFU functions as a diaphorase, and HoxYH functions as a [NiFe]-type hydrogenase. HoxW and HoxI are accessory proteins. SH expression was coupled with the availability of H_2 [14].
(3) The regulatory hydrogenase (RH) consists of the small subunit HoxB and the large subunit HoxC, together forming a tight complex with the histidine protein kinase (HoxJ) [13,14]. HoxA acts as a transcriptional activator of the MBH and SH genes. In the absence of H_2, HoxJ phosphorylates HoxA. The phosphorylated HoxA cannot activate the transcription of MBH and SH. In the presence of H_2, HoxJ cannot phosphorylate HoxA, which makes HoxA able to activate the transcription of the MBH and SH genes [13,14], as illustrated in Fig. 1.
(4) The fourth hydrogenase, an actinobacterial hydrogenase (AH), needs to be better characterized. However, with a relatively slow H_2 consumption rate ($0.5\ s^{-1}$), it presumably functions under low hydrogen concentration conditions [11].

3. Calvin-Benson-Bassham (CBB) cycle for CO_2 fixation in *R. eutropha*

The use of CO_2 as a carbon source is attractive because of its abundance and full detachment from the food industry, which competes for carbon sources. For microbial fermentation, the suitable CO_2 sources include the industrial off-gasses from steel mills and processing plants, the flue gas stream from fossil fuel-fired power stations, and biogas from the conversion of Generation 1 (corn starch) and 2 (lignocellulose) feedstocks to ethanol.

The CBB cycle, also known as the reductive pentose phosphate pathway (PPP), was originally discovered in plants for autotrophic CO_2 fixation [17]. The CBB cycle was reported in diverse eukaryotes and prokaryotes, including *R. eutropha*. In *R. eutropha*, the NADPH and ATP generated through H_2 oxidation by MBH and SH are utilized to produce glyceraldehyde 3-phosphate (G3P) [12]. G3P is channeled into central carbon metabolism (Fig. 1). The CBB cycle, detailed by literature [12], involves 13 enzymatic reactions with reducing powers of 4 equivalents of NAD(P)H and energy in 7 equivalents of ATP.

R. eutropha can grow at high rates on mixtures of H_2, O_2, and CO_2 and accumulate high levels of cell mass [11], with the stoichiometry for autotrophic cell growth shown below [18]:

$$21.4\ H_2 + 6.2\ O_2 + 4.1\ CO_2 + 0.8\ NH_3 \rightarrow C_{4.1}H_{7.1}O_{1.9}N_{0.8} + 18.7\ H_2O \quad (1)$$

Here, the molar ratio of H_2: O_2: CO_2 for gaseous substrate consumption is approximately 5: 1.5: 1. This ratio can change depending on the growth conditions [19].

4. PHB production in *R. eutropha*

R. eutropha is an established host for the commercial production of poly-3-hydroxybutryate (PHB), a polyester that belongs to the family of poly-3-hydroxyalkanoates (PHAs). It has biodegradable and thermoplastic properties [20]. With an excess of carbon and energy, but low concentrations of oxygen and other nutrients that limit growth, G3P can be channeled to produce PHB molecules as energy storage via the intermediate acetyl-CoA for carbon storage in *R. eutropha* H16 [21] (Fig. 1). *R. eutropha* H16 can accumulate PHB to over 80% (w/w) on cell dry weight (CDW) basis [22], with a stoichiometry of PHB as below [23]:

$$33\ H_2 + 12\ O_2 + 4\ CO_2 \rightarrow C_4H_6O_2 + 30\ H_2O \quad (2)$$

Thus, 1.30 kg of PHB can theoretically be produced per kg of H_2 metabolized [22].

The CO_2-rich, industrial off-gasses collected from a bioethanol plant and a biogas plant have been used for the fermentation of *R. eutropha* to produce PHB. The collected CO_2-rich gasses were compressed to 40 bars in 0.05-m^3 bottles. The bottled CO_2 off-gasses, along with H_2 and O_2 were continuously sparged into the bioreactor to reach a constant headspace gas composition of H_2:O_2:CO_2, which equals 84: 2.8: 13.2 vol%. These fermentations achieved a high biopolymer content (up to 73%) and productivity (up to 0.227 g/L h) [24].

5. 2,3-butanediol (2,3-BDO) pathway in engineered *R. eutropha* H16

As an economically important bulk chemical, 2,3-BDO can be used for chemical synthesis and conversion into various products, such as synthetic rubber, solvents, food additives, gasoline, diesel, and jet fuel [25]. In the decade of 2010–2020, *R. eutropha* has been engineered by blocking the PHB production pathway, whereby the reducing equivalents and carbon flux are redirected to produce a broad range of chemicals, including acetoin, alkanes, *β*-farnesene, 2-hydroxyisobutyrate, isobutanol, and

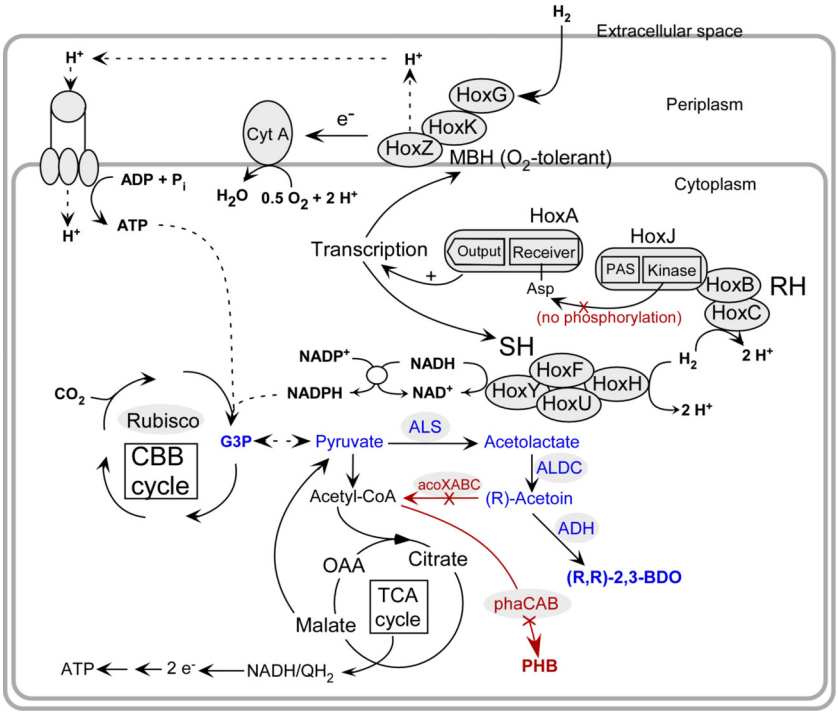

Fig. 1. The central metabolism and key enzymes for synthesizing (R,R)-2,3-BDO in *R. eutropha* H16. The diagram is prepared based on literature that describes the membrane-bound hydrogenase (MBH) complex [5,12,15] and the 2,3-BDO pathway [16]. The reported, engineered strain of *R. eutropha* H16 converts CO_2 and H_2 to pyruvate via the Calvin Cycle and redirects carbon flux from pyruvate to (R,R)-2,3-BDO. The red lines depict the deleted endogenous PHB pathway, whereas the blue lines depict the heterologous expression of the 2,3-BDO pathway. Abbreviations for enzymes and metabolites are given in the Abbreviation list.

methyl ketones [6]. More recently, the natural carbon sink pathway *R. eutropha* strain H16 to PHB was blocked by knocking out the operon phaC1AB1, thus redirecting reducing equivalents and carbon flux to the formation of 2,3-BDO [16].

The heterologous pathway for the synthesis of 2,3-BDO used by *R. eutropha* H16, as illustrated in Fig. 1, consists of three enzymatic steps: **(1)** pyruvate conversion to 2-acetolactate by the 2-acetolactate synthase (ALS) from *Bacillus subtilis*; **(2)** 2-acetolactate decarboxylation to stereospecific (R)-acetoin by acetolactate decarboxylase (ALDC) from *B. subtilis*; and **(3)** (R)-acetoin was reduced to 2,3-BDO by an NADPH-specific alcohol dehydrogenase (ADH) from *Clostridium beijerinckii*. The 2,3-BDO-pathway genes were overexpressed as a single operon via chromosomal integration. In addition to phaC1AB1 (corresponding to the PHD pathway), the acoXABC gene cluster that is responsible for the degradation of the BDO precursor, acetoin, was also deleted in the 2,3-BDO-producing strain [16].

Continuous fermentations (up to nearly 500 h) using *R. eutropha* strains producing 2,3-BDO have been reported [16], using a DASGIP® bioreactor system, 1-L capacity vessels with a 750-mL working volume, and separate feeds for air, CO_2, and H_2. A specific, steady-state CO_2 uptake rate of 3 (mmol C)/(gDCW·h) was observed. The CO_2 was reduced to carbon sinks that include the fermentation product (R, R)-2,3-BDO, cell mass, and by-products (i.e., meso-2,3-butanediol in this case), resulting in high carbon efficiency of 0.75 C-mol (R, R)-2,3-BDO/C-mol CO_2, with 0.21 C-mol cell mass/C-mol CO_2 and 0.07 C-mol Meso-2,3-BDO/C-mol CO_2 representing the remaining balance of carbon. In addition, they reported a titer of 32 g/L for the product (R, R)-2,3-BDO in the liquid phase of fermentation. However, their results indicated a poor energy efficiency of 8 mol H_2/mol CO_2, generating significant heat from the exothermic reaction and limiting the economic feasibility of the process. Promisingly, this inefficiency could be overcome through process integration with the H_2 supply produced from endothermic supercritical H_2O gasification that functioned as a heat sink for the process of exothermic gas fermentation [12,16].

It is noteworthy that a recent study reported the engineering of *R. eutropha* H16 for the production of (R)-1,3-BDO from CO_2, which achieved a titer of 2.97 g/L of (R)-1,3-BDO autotrophically [26], much lower than the 32 g/L titer cited above [16]. In both cases, future engineering of BDO-producing *R. eutropha* strains is needed to improve the titer and production rates of BDO, regardless of the chemical forms of BDO (1,3- or 2,3-). In addition, *R. eutropha* showed reduced tolerance and impaired growth rate to BDO with a concentration above 30 g/L. Future studies are needed to address this issue.

6. 2,3-BDO production in engineered *Z. mobilis* strains

Z. mobilis is an ethanologen that is well known for its high specific sugar uptake rate and rapid catabolism and has been engineered to metabolize all major biomass sugars [27]. In 2016, our group demonstrated that carbon flux could be directed away from ethanol production to 2,3-BDO biosynthesis by expressing all three heterologous 2,3-BDO pathway genes. These genes encode **(1)** acetolactate synthase (ALS), **(2)** acetolactate decarboxylase (ALDC), and **(3)** butanediol dehydrogenase (BDH) [28], as illustrated in Fig. 2. The best performing strain, 9C-BC11, was generated by transforming ethanol-producing *Z. mobilis* strain 9C with the plasmid pEZ-BC11 that carried **(1)** *B. subtilis Als* gene driven by inducible promoter Ptet (Ptet-BsAls), and **(2)** *aldC* and *bdh* gene operons from *E. cloacae* driven by the strong promoter, Pgap[i.e. Pgap-(EcAldc-Bdh)]. The 9C-BC11 strain achieved titers of 13 g/L 2,3-BDO, 0.7 g/L acetoin, and 25 g/L ethanol with 40 mL RMG8 medium containing 200 μg/mL spectinomycin, without tetracycline induction, in 125-mL flask at 33 °C with 120 rpm. These results also indicated Ptet is a leaky promoter in *Z. mobilis* strains. To boost the 2,3-BDO production titer, there is ongoing work by our group to optimize the genetic components in expressing the 2,3-BDO pathway genes and to either reduce or block the production of side products.

7. Proposal for introducing CO_2 and H_2 utilizing pathways into 2,3-BDO-producing *Z. mobilis* strains

Although the above 2,3-BDO-producing *Z. mobilis* strains had been used to alleviate rising atmospheric CO_2 concentration by fermenting hydrolysate sugars derived from corn stover, they are heterotrophic and

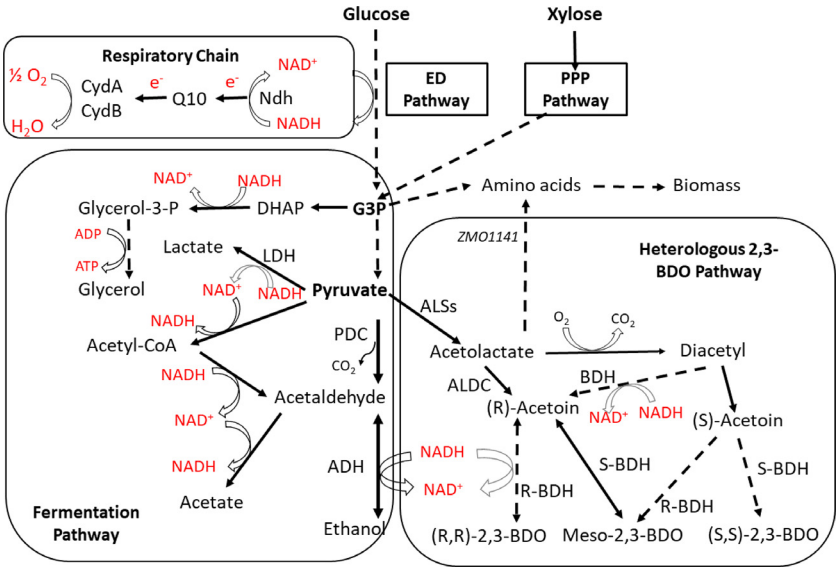

Fig. 2. A scheme for the heterologous 2,3-BDO biosynthesis pathway that was integrated into the *Z. mobilis* native central carbon metabolism [28], which includes the PPP (pentose phosphate pathway), ED (Entner–Doudoroff), and fermentation pathways for the production of ethanol and minor metabolites such as acetate, lactate, and glycerol from glucose and xylose. In the illustrated heterologous 2,3-BDO pathway, the dashed lines indicate the possible steps; ALSs include both the expressed *B. subtilis* acetolactate synthase and the endogenous homologs of *Z. mobilis*, including ZMO1139, ZMO1140, and ZMO0687. In other illustrated pathways, the dashed lines indicate multiple steps. Abbreviations for enzymes and metabolites are given in the Abbreviation list.

cannot use CO_2 as substrate directly. Thus, we propose to engineer *Z. mobilis* to mimic *R. eutropha*'s CBB cycle and H_2 utilization pathway to enable an autotrophic growth of *Z. mobilis* for 2,3-BDO production.

R. eutropha H16 has a shorter CBB cycle than plants [29], making it a promising candidate to be heterologously expressed in non-CO_2-fixing microorganisms. Indeed, Rubisco and phosphoribulokinase from *R. eutropha* H16 have been co-expressed in yeast *S. cerevisiae* for in situ fixation of CO_2 and the production of ethanol [30]. Such studies support the feasibility of expressing *R. eutropha* H16's Rubisco in other heterotrophic microorganisms.

Furthermore, a recent study showed that the CBB pathway could be engineered in *C. necator* H16 by expressing a heterologous Rubisco from a cyanobacterium, *Synechococcus* sp. PCC 7002, coupled with the increased expression of membrane-bound and soluble hydrogenases via the insertion of a heterologous, strong promoter from *E. coli* [31]. The resulting strain had a 93.4% increase in cell growth and a 99.7% increase in PHB accumulation compared to the control strain, *C. necator* H16(pBAD-RFP) [31]. Thus, the above cyanobacterial Rubisco from *Synechococcus* can be a promising alternative candidate gene to be expressed in *Z. mobilis*.

8. Advantage for using 2,3-BDO-producing *Z. mobilis* strains to express CO_2 fixation pathway: excess NADH and partial pentose phosphate pathway

For the reported 2,3-BDO-producing *Z. mobilis* strains, one challenge remains. The synthetic BDO pathway implemented into the engineered strain produces extra NADH, which needs to be oxidized to reduce its toxicity to the cells [28]. The proposed overexpression of the heterologous CO_2 fixation pathway could help rebalance the redox in cells. The excess NADH can be used as a reducing power for the proposed integration of heterologous CO_2-fixation pathway into *Z. mobilis* strains.

As described in the early section (i.e. Calvin-Benson-Bassham cycle for CO_2 fixation in *R. eutropha*), the CBB cycle is also known as the reductive pentose phosphate pathway. The detailed PPP in *R. eutropha* has been described in the literature [12]. Notably, a partial PPP exists in *Z. mobilis* [28,32]. A comparative analysis of the PPP between *R. eutropha* and *Z. mobilis* would identify the overlay enzymes between these two species, which could be used as shared enzymes between the endogenous PPP pathway and the proposed, to-be-expressed heterologous CBB pathway in *Z. mobilis*, facilitating the engineering efforts aiming for autotrophic *Z. mobilis*.

9. Challenges for enabling *Z. mobilis* to utilize CO_2 and H_2: ED and Embden-Meyerhof-Parnas (EMP) pathways and ATP production

Z. mobilis uses the ED pathway in glycolysis, by which only one net ATP is generated for each glucose molecule consumed, as illustrated in Fig. 3. Protein IDs of the enzymes in the ED pathway are based on published literature [33]. This ED pathway provides less energy for growth and maintenance than the EMP pathway in other bacteria that generates a net of two to three ATP per glucose molecule consumed, depending on if ATP-dependent or PPi-dependent phosphofructokinase (PFK) is used. However, the EMP pathway is incomplete in *Z. mibilis*, as illustrated by the dashed lines for the missing reaction steps in Fig. 3.

Studies explored the possibility that the ED pathway in *Z. mobilis* limits its ATP production and growth by aiming to complete an EMP glycolysis pathway through the heterologous expression of either a PPi-dependent PFK [34], or an ATP-dependent PFK [35], alone or in combination with fructose bisphosphate aldolase (FBA) and triosephosphate isomerase (TPI), as illustrated in Fig. 3. Both studies showed that the introduction of EMP enzymes was unable to complete an EMP pathway. However, some shifts in the metabolism [34] or inhibition of cell growth [35] were observed, suggesting that the homeostatic levels of glycolytic intermediates in *Z. mobilis* might be incompatible with the EMP flux [35]. To meet the high ATP demand for proposed CO_2 assimilation in *Z. mobilis*, future studies are needed to:

(1) Downregulate the abundantly expressed ED pathway enzymes to mitigate the burden that the expression of ED-pathway proteins may cause.
(2) Further engineer *Z. mobilis* to complete the EMP pathway by selecting and expressing more compatible EMP enzymes.
(3) Genetically modify the PPP pathway in *Z. mobilis*, to reach a state of balance among ED, EMP, and PPP pathways, which could enable CO_2 reduction in *Z. mobilis*.

To provide the necessary amount of ATP for CO_2 fixation, the above-proposed putative recombinant *Z. mobilis* strain may still need to metabolize a significant amount of glucose, thus leading to a mixed fermentative/autotrophic metabolism in the proposed strains.

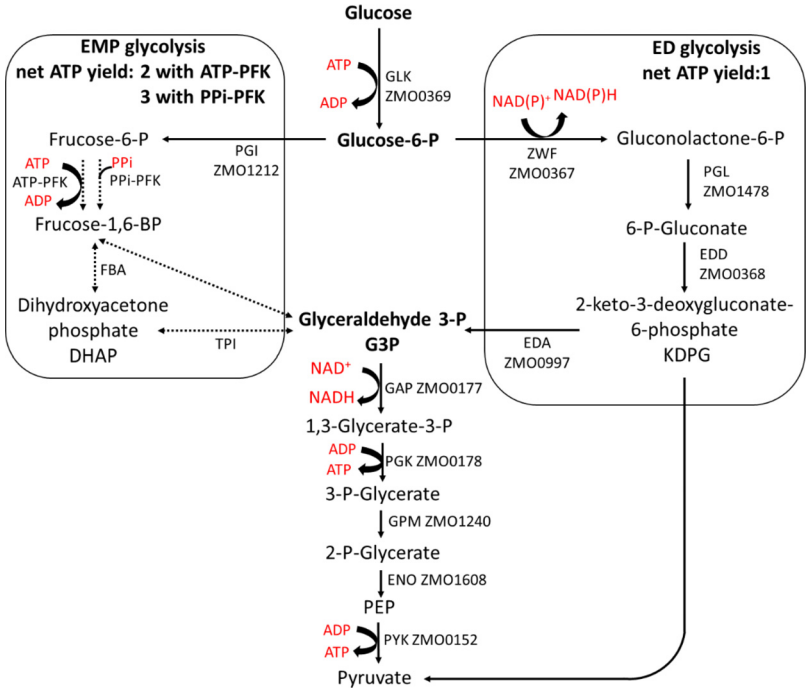

Fig. 3. Scheme of the Entner–Doudoroff (ED) and incomplete Embden-Meyerhof-Parnas (EMP) glycolysis pathways in *Z. mobilis*. The dotted lines indicate the reactions that are absent in its EMP pathway.

10. Challenges and knowledge gaps for engineering *Z. mobilis* to utilize CO_2 and H_2: soluble hydrogenase system, CO_2 concentrating mechanisms, and carbonic anhydrases

The *R. eutropha* SH system consists of at least 12 proteins, which include hoxFUYHWI (among which hoxFUYH is illustrated in Fig. 1), HypABCD, and HypEF (i.e., maturase and chaperone complexes) [11,36]. The heterologous expression of *Ralstonia* SH in heterotrophic hosts was recently reviewed [37]. The multicomponent nature of *R. eutropha* SH, its complicated maturation and regulation processes, and the high specificity of these maturation proteins make the heterologous production of oxygen-tolerant hydrogenases a challenge, as exemplified by the co-expression of RH from *R. eutropha* [38–40]. In addition, heterologous expression of these 12 SH system-related genes in *Z. mobilis* will likely form a significant metabolic burden on the host cells. Thus, an alternative, simplified SH system may be needed to engineer *Z. mobilis* strains.

The K_m value of the Rubisco enzyme for CO_2 (K_c) in *R. eutropha* was estimated to be 34 μM from anaerobic assays and 66 μM from aerobic assays [41], which are in a range comparable to those of Rubisco obtained from plants and marine diatoms [41, 42]. In general, an increase in substrate concentration can improve the thermodynamics and efficiency of enzyme function. For microbial CO_2 fixation, CO_2 concentrating mechanisms (CCMs) such as carboxysomal carbonic anhydrases (CA) play an important role in minimizing the imbalance between low intracellular CO_2 concentrations and increasing demands of inorganic carbon [43].

CA enzymes catalyze the interconversion between CO_2 and bicarbonate (HCO_3^-). *R. eutropha* H16 was found to have four CA genes: *can, can2, caa, and cag*, with specific activity in the range of 60 to 422 EU mg^{-1}. Deletion of *can* (encoding a β-CA) and *caa* (encoding an α-CA) detrimentally affected cell growth [44]. In addition, while CO_2 can freely diffuse into cells, $HCO3^-$ can only be taken up by Na^+ or ATP-dependent transporters [45]. Thus, studies are needed to examine the genes encoding CAs and HCO_3^- transporters in the genome of *Z. mobilis* and investigate the dynamics between the external and intracellular CO_2. Such studies would likely facilitate the proposed expression of heterologous CO_2 fixation pathway in *Z. mobilis*.

11. Conclusions

Microbial metabolic conversion of CO_2 to essential bio-based chemicals is a promising route to address the challenges of a rising atmospheric CO_2 concentration. This review focuses on recent progress using *R. eutropha* to convert CO_2 to 2,3-BDO. The intrinsic limitation caused by the intolerance of the host strain to BDO renders its current maximal BDO titer (~30 g/L) by continuous fermentation economically infeasible for commercialization. Thus, future studies for improving CO_2 fixation by expressing heterologous, more efficient Rubisco enzymes and implementing carbon concentration mechanisms aiming for increasing BDO titers in *R. eutropha* are warranted.

Moreover, the remarkably high utilization rate of sugars and the production of 2,3-BDO from engineered *Z. mobilis* strains provide an attractive alternative model microorganism to focus on. For example, new works should expand its substrate spectrum by expressing heterologous CO_2-fixing and H_2-splitting pathways and implementing carbon-concentrating mechanisms, to enable *Z. mobilis* for autotrophic and mixotrophic growth and for the direct utilization of CO_2 and H_2 in producing 2,3-BDO.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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