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Research review paper

Optimization strategies for CO_2 biological fixation

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ABSTRACT

Global sustainable development faces a significant challenge in effectively utilizing CO_2. Meanwhile, CO_2 biological fixation offers a promising solution. CO_2 has the highest oxidation state (+4 valence state), whereas typical multi-carbon chemicals have lower valence states. The Gibbs free energy (ΔG) changes of CO_2 reductive reactions are generally positive and this renders it necessary to input different forms of energy. Although biological carbon fixation processes are friendly to operate, the thermodynamic obstacles must be overcome. To make this reaction occur favorably and efficiently, diverse strategies to enhance CO_2 biological fixation efficiency have been proposed by numerous researchers. This article reviews recent advances in optimizing CO_2 biological fixation and intends to provide new insights into achieving efficient biological utilization of CO_2. It first outlines the thermodynamic characteristics of diverse carbon fixation reactions and proposes optimization directions for CO_2 biological fixation. A comprehensive overview of the catalytic mechanisms, optimization strategies, and challenges encountered by common carbon-fixing enzymes is then provided. Subsequently, potential routes for improving the efficiency of biological carbon fixation are discussed, including the ATP supply, reducing power supply, energy supply, reactor design, and carbon enrichment system modules. In addition, effective artificial carbon fixation pathways were summarized and analyzed. Finally, prospects are made for the research direction of continuously improving the efficiency of biological carbon fixation.

1. Introduction

The "greenhouse effect" gradually intensifies as many greenhouse gases are released. The United Nations Secretary-General Antonio Guterres said, "The era of global warming has ended; the era of global boiling has arrived." Solving the problem of the "greenhouse effect" is urgently required. The greenhouse gases include CO_2, methane, N_2O, and other greenhouse gases. The proportion of CO_2 in greenhouse gases has increased to 76.7% (*v*/v), making it the primary cause of climate change (Kajla et al., 2022). Notably, the current CO_2 level in the atmosphere is almost twice that of the preindustrial period. Fig. 1 shows the change in global CO_2 concentration, annual growth rates, and average growth every five years over the last 40 years. This result shows that the CO_2 levels are increasing steadily and that the increase is accelerating. The accumulation of CO_2 has long-term adverse consequences, including glaciers decrease, permafrost melt, ocean acidification, snowpack decrease, etc. Therefore, controlling and decreasing CO_2 leaves has become a primary concern.

One strategy for achieving this aim is to use biological systems to convert CO_2 into multi-carbon compounds. This approach reduces CO_2 emissions while providing a free carbon source for the biosynthesis of multi-carbon products, resulting in a win-win situation. The biological conversion of CO_2 has garnered much interest due to its gentle process conditions, eco-friendly approach, and product selectivity. The development of biological CO_2 fixation research has led to the discovery of natural CO_2 fixation pathways for constructing artificial CO_2 fixation pathways. Until 2021, six natural CO_2-fixation routes have been reported. These pathways can be separated into aerobic and anaerobic CO_2-fixation pathways, depending on the presence of specific oxygen-sensitive enzymes. Aerobic pathways include the Calvin cycle (the CBB cycle), the 3-hydroxypropionate (3HP) cycle, and the 3-hydroxypropionate-4-hydroxybutyrate (3HP/4HB) cycle. Anaerobic pathways comprise the reductive tricarboxylic acid (rTCA) cycle, the Wood-Ljungdahl (WL) pathway, and the dicarboxylate/4-hydroxybutyrate (DC/4HB) cycle. However, natural CO_2-fixation pathways are energy intensive or thermodynamically unfavorable (Zhao et al., 2021).

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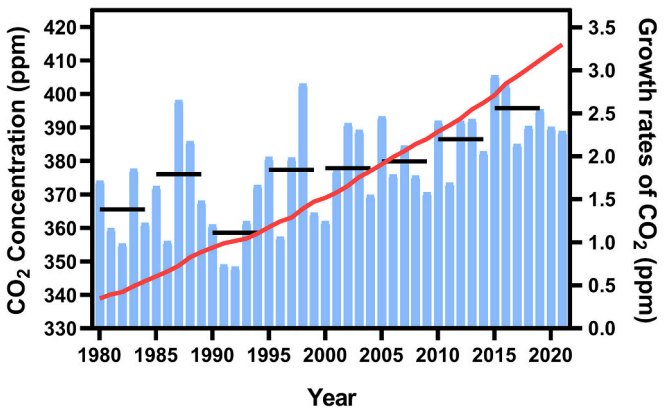


Fig. 1. The global average concentration of CO_2 (1980–2021). The histogram shows the annual growth rates of CO_2. The red curve shows the annual CO_2 concentration. The black line shows the average growth rate of CO_2 every five years. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Therefore, artificial CO_2-fixation pathways with improved efficiency were designed. Artificial CO_2-fixation pathways can be divided into two categories according to the properties of critical carbon-fixing enzymes: carboxylase-mediated synthetic CO_2-fixation pathways and reductase-mediated synthetic CO_2-fixation pathways(Jiang et al., 2020). The carboxylase-mediated synthetic CO_2-fixation pathways include malonyl-CoA-oxaloacetate-glyoxylate pathway (MOG), malonyl-CoA-glycerate pathway (MCG), crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyrate-CoA cycle (CETCH), and POAP cycle. Reductase-mediated synthetic CO_2-fixation pathways include the reductive glycine pathway (rGly), formolase pathway, synthetic acetyl-CoA pathway (SACA), and artificial starch anabolic pathway (ASAP).

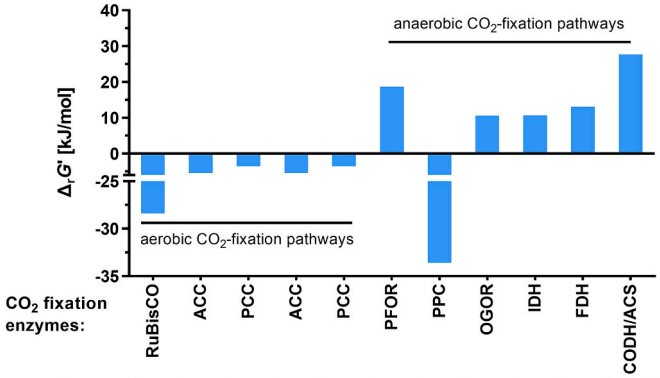
In CO_2 bioconversion process, CO_2 first enters carbon fixation pathways at different levels. After a series of metabolic reactions, CO_2 can then be converted into reducing intermediate metabolites, such as pyruvate or acyl-CoA. These products can be used as critical metabolic intermediates for cell growth or as building blocks to produce long-chain carbon compounds. CO_2 molecules have the highest oxidation state (+4 valence state), whereas typical multi-carbon chemicals (hydrocarbons, aldehydes, acids, or alcohols) have lower valence states. Consequently, the Gibbs free energy (ΔG) changes of CO_2 reductive processes are generally positive and this makes it necessary to input different forms of energy for carbon fixation. Up to now, various designs to improve CO_2 fixation efficiency of autotrophic and heterotrophic microorganisms have been reported. This review discusses the latest progress in optimizing CO_2 biological fixation. Initially, it examined the thermodynamic properties of carbon fixation reactions and proposed optimization directions to enhance their efficiency. Subsequently, the catalytic mechanisms and optimization techniques employed by core carbon fixation enzymes are summarized. Additionally, it focuses on optimization strategies for ATP, reducing power, energy supply modules, reactor design, and carbon enrichment systems. Recent advances in artificial carbon fixation pathways have also been described. Finally, the perspectives of CO_2 biological fixation are discussed.

2. Thermodynamic characterization of carbon fixation reactions

Carbon fixation enzymes are key limiting factors in carbon fixation pathways. Although the enzyme can reduce the reaction activation energy, it does not alter the thermodynamic equilibrium. Therefore, it is necessary to understand the thermodynamic characteristics of carbon fixation reactions, which may provide guidance for the optimization of biological carbon fixation processes. Among the six natural carbon fixation pathways, there are nine carbon fixation enzymes. The six natural carbon fixation pathways have been reviewed in many publications and are therefore not presented here (Gong et al., 2016). In this section, we analyze the thermodynamic characteristics of the nine critical carbon fixation reactions and discuss optimization strategies.

Aerobic CO_2-fixation pathways have three carbon fixation enzymes: acetyl-CoA carboxylase (ACC), propionyl-CoA carboxylase (PCC), and ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO). Among them, the carbon fixation reactions catalyzed by ACC (3HP and 3HP/4HB cycles) and PCC (3HP and 3HP/4HB cycles) take HCO_3^- as the reaction substrate. When there is no energy input, the reaction changes in Gibbs free energy ($\Delta_r G$) are above 0 (38.1 ± 6.5 and 39.9 ± 11.8 kJ/mol, respectively. $\Delta_r G$ values were all calculated at pH 7.0, ionic strength of 0.1 M, and substrate concentration of 1 mM), which are thermodynamically challenging. However, with the input of energy (ATP), the reaction values of $\Delta_r G$ are <0 (−5.4 ± 6.5 and − 3.6 ± 1.8 kJ/mol, respectively), making it thermodynamically conducive to carbon fixation. Additionally, Zhao et al. indicated that $\Delta_r G$ values would be altered more negatively with increased ATP input, demonstrating that intracellular ATP availability is crucial for thermodynamic feasibility (Zhao et al., 2021). Therefore, a higher input of ATP would be a wise option to improve the carbon fixation catalyzed by ACC and PCC. Another carbon fixation enzyme, RuBisCO (present in the Calvin–Benson–Bassham (CBB) cycle), could catalyze CO_2 addition to RuBP (ribulose-1,5-bisphosphate), producing 3PG (3-phosphoglycerate). The $\Delta_r G$ of the reaction catalyzed by RuBisCO is less than zero (−85.1 ± 22.4 kJ/mol) without energy and reducing force. This thermodynamically advantageous phenomenon occurs because RuBP is a high-energy-state substrate (Zhao et al., 2021). The synthesis reaction of RuBP, utilizing Ru5P as substrate, requires the input of ATP. The $\Delta_r G$ of this reaction is <0 (−56.6 ± 13.5 kJ/mol), which is lower than the $\Delta_r G$ values of substrates formation reactions involving other carbon fixing enzymes (e.g., $\Delta_r G$ of succinyl-CoA formation is −1.8 ± 2.6, $\Delta_r G$ of acetyl-CoA formation is −7.9 ± 15.3). Despite the ATP-independent nature of the carbon-fixing reaction catalyzed by RuBisCO, its coupling with the synthesis reaction of its substrate RuBP (ATP-dependent) still provides thermodynamic advantages for RuBisCO. However, the RuBisCO active sites also catalyze RuBP oxygenation. When RuBisCO is exposed to O_2 or the intracellular CO_2 concentration is low, O_2 competes with CO_2 for the active site of RuBisCO and triggers the photorespiration process. This strong side reaction leads to losing up to 30% of photosynthetic fixed carbon. Therefore, a higher intracellular CO_2 concentration would be an excellent choice for improving the RuBisCO-catalyzed carbon fixation process and increasing the efficiency of carbon bioconversion.

Anaerobic CO_2-fixation pathways comprise six carbon fixation enzymes: phosphoenolpyruvate carboxylase (PPC), pyruvate: ferredoxin oxidoreductase (PFOR), oxoglutarate ferredoxin oxidoreductase (OGOR), isocitrate dehydrogenase (IDH), formate dehydrogenase (FDH), and the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). Among them, the CO_2 fixation reactions catalyzed by FDH (WL pathway), CODH/ACS (WL pathway), IDH (rTCA cycle), OGOR (rTCA cycle), and PFOR (DC/4HB cycle) are ATP-independent but depend on NADPH or reduced ferredoxin. Unlike aerobic pathways, the $\Delta_r G$ values of these CO_2-fixation reactions in anaerobic strains tended to be higher than 0 (Fig. 2). It indicates that these CO_2-fixation reactions are relatively unfavorable and thermodynamically challenging. However, these carbon fixation reactions can still occur in natural anaerobic carbon fixation pathways. The main reason is that these carboxylation reactions ($\Delta_r G > 0$) are coupled with other reactions (most $\Delta_r G$ values are negative), making the $\Delta_{responses}$ of the whole pathway <0. Meanwhile, the additional energy and reducing equivalents input ($\Delta_r G$ of ATP hydrolysis, NADPH oxidation, and reduced ferredoxin are −43.5 ± 0.6, −65.1 ± 1.2, and − 40.4 ± 5.8 kJ/mol, respectively) would facilitate the carbon fixation process. For example, PFOR catalyzes the generation of pyruvate from acetyl-CoA and CO_2 in the DC/4HB cycle. The $\Delta_r G$ value of this reaction is above 0, indicating that it is thermodynamically unfavorable. To favor the forward reaction, the carbon fixation product



ATP	0	1	1	1	1	0	0	0	0	0	0
NADPH	0	0	0	0	0	0	0	0	1	1	0
Fd_{red}	0	0	0	0	0	2	0	2	0	0	2
pathway	CBB	3HP	3HP	3HP/ 4HB	3HP/ 4HB	DC/ 4HB	DC/ 4HB	rTCA	rTCA	WL	WL

Fig. 2. Alignment of different CO_2 fixation enzymes. RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; ACC, acetyl-CoA; PCC, propionyl-CoA carboxylase; PFOR, pyruvate: ferredoxin oxidoreductase; PPC, phosphoenolpyruvate carboxylase; OGOR, oxoglutarate ferredoxin oxidoreductase; IDH, isocitrate dehydrogenase; FDH, formate dehydrogenase; CODH/ACS, the CO dehydrogenase/acetyl-CoA synthase; Fd_{red}, reduced ferredoxin; CBB, the Calvin cycle; 3HP, the 3-hydroxypropionate cycle; 3HP/4HB, the 3-hydroxypropionate-4-hydroxybutyrate cycle; DC/4HB, the dicarboxylate/4-hydroxybutyrate cycle; rTCA, the reductive tricarboxylic acid cycle; WL, the Wood-Ljungdahl pathway.

pyruvate must therefore be consumed quickly. In addition, the Δ_rG values of the next several reactions of this step are far <0, which can help drive the reaction catalyzed by PFOR toward the desired direction. Therefore, although the Δ_rG of a certain reaction may be positive, the reaction can still be driven when the Δ_rG of the whole pathway is negative. Accordingly, when constructing the artificial carbon fixation pathways around these five CO_2 fixation enzymes, there are potential approaches that can be implemented to improve the system's performance. First, improving intracellular ATP or reducing equivalents availability may be helpful. Second, ensure the Δ_rG of whole process is <0. From a thermodynamic perspective, the pathway with a more significant negative Δ_rG value has a more vital driving force and a robust system. Furthermore, only the Δ_rG of the reaction catalyzed by PPC (DC/4HB cycle) is negative (-33.6 ± 6.4 kJ/mol) among the anaerobic CO_2-fixation pathways. PPC converts HCO_3^- and phosphoenolpyruvate into oxaloacetate and orthophosphate. Notably, this reaction is ATP-independent and reducing equivalents-independent. The reason for this thermodynamic advantage is that phosphoenolpyruvate (PEP) is a high-energy phosphate compound. In the DC/4HB cycle, PEP is synthesized from pyruvate as the substrate, in which ATP is utilized, resulting in high-energy phosphate bonds in PEP. The Δ_rG for this reaction is negative, specifically -13.3 ± 1 kJ/mol, which is comparatively lower than the Δ_rG values observed in substrate formation reactions of other carbon-fixing enzymes. Despite the ATP-independent and reducing power-independent nature of the reaction catalyzed by PPC, the coupling of this reaction with its substrate PEP synthesis reaction (ATP-dependent) still confers thermodynamic advantages for PPC. PPC has been extensively used in fuel and chemical biosynthesis owing to its favorable thermodynamic properties. For example, Tan et al. (Tan et al., 2013b) designed a PPC-based succinate biosynthetic route, which can fix one molecule of CO_2 to produce one succinate molecule. The wide application of PPC provides a green route for the direct conversion of CO_2 to many building blocks and provides references for the application of other carbon fixation enzymes.

Currently, the six known natural CO_2 fixation pathways are thermodynamically feasible. In general, the reactions in aerobic strains are thermodynamically favorable but energy-intensive for the main carbon fixation steps. Conversely, the reactions in anaerobic strains are more energy-saving but thermodynamically unfavorable and require reducing equivalents. Thermodynamic analysis also provides valuable insights to overcome the inert nature of CO_2 and realize its high-efficiency bioreduction. These include identifying and selecting carbon-fixing enzymes to efficiently reduce reaction activation energy, optimizing ATP or reducing power supply modules in CO_2 fixation, or developing artificial or hybrid carbon fixation pathways with superior thermodynamic efficiency.

3. Advances in carbon fixation enzymes

The carbon fixation enzyme is critical for the biological fixation of CO_2. Choosing an efficient CO_2 fixation enzyme is beneficial for reducing the reaction activation energy, and can accelerate the ligation of CO_2 to particular substrates. The presence of a robust chemical inertness in CO_2 necessitates the surmounting of a considerable activation energy for substrate nucleophilic attack on CO_2. Carbon fixation enzymes frequently employ a distinctive reaction mechanism to maintain the substrate in a profoundly active and unstable state, while concurrently stabilizing the transition state of the reaction through the interaction between the enzyme and the substrate. This reduce the activation energy. Consequently, a comprehensive understanding of the catalytic mechanism of carbon-fixing enzymes will aid in elucidating the activation mechanism of CO_2, thereby establishing a theoretical foundation for screening and optimizing carbon fixation enzymes. The six natural carbon fixation pathways included nine carbon fixation enzymes: ACC, PCC, RuBisCO, PPC, PFOR, OGOR, LDH, FDH, and CODH/ACS. Typically, only the Δ_rG values of the reactions catalyzed by ACC, PCC, PPC, and RuBisCO were <0. These enzymes have been intensively studied because of their excellent catalytic performance and widespread application. This section focuses on the catalytic mechanism and optimization strategies for ACC, PCC, PPC, and RuBisCO.

3.1. Acetyl-CoA carboxylase (ACC) and propionyl-CoA carboxylase (PCC)

ACC (EC 6.4.1.2) and PCC (EC 6.4.1.3) were the core carbon-fixing enzymes in the 3HP and 3HP/4HB cycles. ACC catalyzes acetyl-CoA carboxylation with bicarbonate, producing malonyl-CoA, and PCC catalyzes propionyl-CoA carboxylation with bicarbonate, creating methylmalonyl-CoA. ACC and PCC belong to a subgroup of biotin-dependent short-chain acyl-CoA carboxylases. They share a similar structure consisting of biotin carboxylase domains (BC), carboxyltransferase domains (CT), and biotin-carboxyl carrier protein domains (BCCP), and utilize covalently bound biotin as a cofactor. ACC and PCC involve two distinct enzymatic activities and complete catalytic reactions in two steps. First, biotin covalently binds to the BCCP domain active site through an amide bond. The BC domain then catalyzes MgATP-dependent carboxylation of the N1 atom of biotin using bicarbonate as the CO_2 donor. Second, the CT domain catalyzes the carboxyl group transfer from carboxy-biotin to the α-carbon of the substrate acyl-CoA (Fig. 3**A**). BCCP and BC domains are conserved among ACC and PCC, but the CT domain is highly distinct. The CT domain could recognize the CoA segment and determine the reaction specificity. Without the CT domain, BC-BCCP-biotin complexes show little to no catalytic activity unless they are complexed with CT domain and acetyl-CoA or propionyl-CoA. Broussard et al. (Broussard et al., 2013) reported that the activity of the pure BC-BCCP complex was 0.005 μmol/min/mg. However, in the presence of the CT domain and acetyl-CoA, the BC-BCCP activity increased significantly (up to 0.53 μmol/min/mg). This indicates that classic substrate synergism is a catalytic characteristic of ACC and PCC. It is important for the regulation of ACC or PCC activity and it prevents the wasteful hydrolysis of ATP unless all substrates are present.

There are several strategies for enhancing ACC and PCC activity. i)

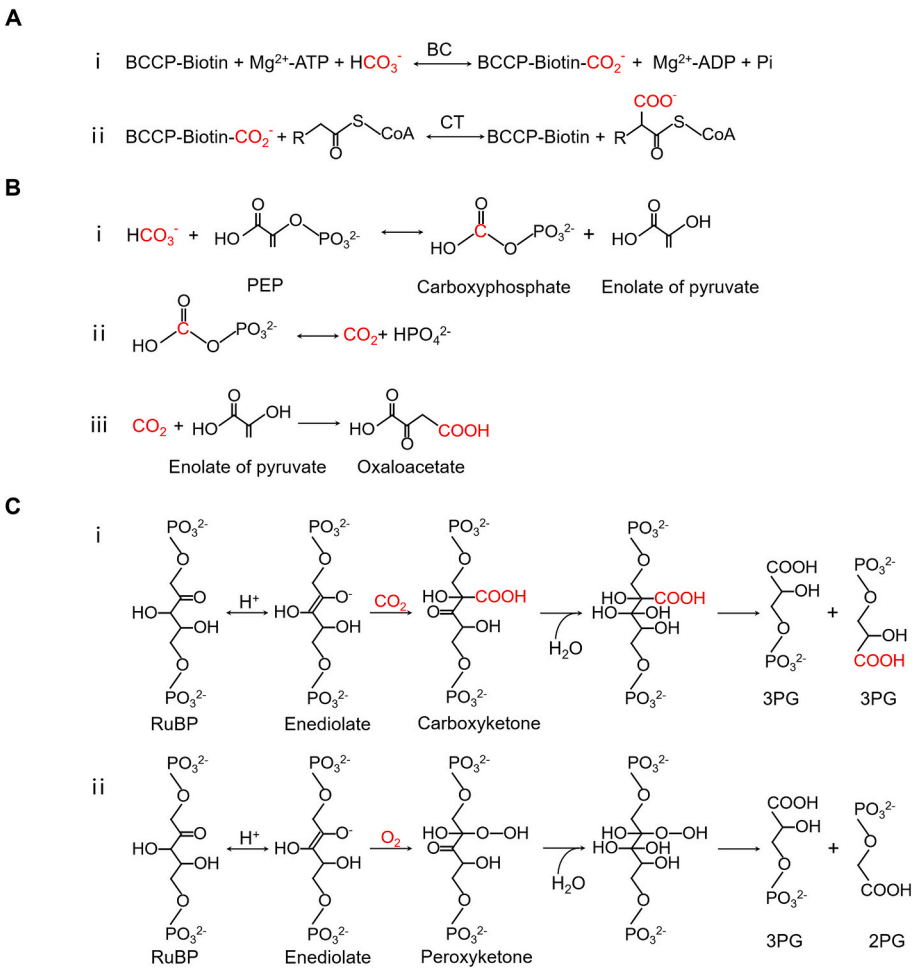


Fig. 3. Catalytic mechanism of different carbon fixation enzymes. A: The catalytic process of PCC, which is present in bacteria, fungi, or archaea. BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; CT, carboxyltransferase. B: The catalytic process of PPC, which is present in bacteria, fungi, or archaea. C: The catalytic process of RuBisCO, which is present in plants or algae. i: Carboxylation process of RuBisCO; ii: Oxidation process of RuBisCO.

Manipulating enzyme levels through gene amplification. Davis et al. (Davis et al., 2000) overexpressed a set of four ACC subunit proteins in a coordinated manner, leading to a nearly 50-fold increase in ACC activity. ii) Engineering the protein at post-translational level. Scott et al. (Scott et al., 2002) found that glucose deprivation negatively affected ACC through AMP-activated serine/threonine protein kinase (Snf1). ACC is deactivated when Snf1 phosphorylates one or more serine residues in ACC. Shi et al. (Shi et al., 2014) showed that mutants lacking Snf1 display increased ACC activity. Nevertheless, Snf1 is also related to gluconeogenesis, glyoxylate cycle, and β-oxidation of fatty acids. Therefore, it is impossible to remove Snf1 to enhance ACC activity of ACC. Jin et al. (Jin and Silva, 2014) aligned different sources of ACC and identified Ser-1157 as the critical amino acid for phosphorylation and deactivation. Moreover, they mutated Ser-1157 to Ala-1157 to optimize ACC, resulting in 9 times higher specific activity than wild-type ACC. Except for Ser-1157, Shi et al. (Shi et al., 2014) proposed another putative phosphorylation site (Ser-659) of ACC. They showed that the S659A mutation was also functional and led to a 1.72-fold increase in ACC activity. This suggests the need for engineering ACC at the post-translational level to regulate its activity. iii) Enzyme engineering. Liu et al. (Liu et al., 2020a) tested several CT domains of various microorganisms to obtain PCC with high enzyme activity and found CT domain from *Bacillus subtilis* showed the highest activity in vitro. They also designed a genetic screening system based on oxaloacetate availability to further optimize PCC. A highly active PCC mutant (N220I/I391T) was obtained, which showed a 5.6-fold increase compared to the wild-type. This also indicates the potential application of enzyme engineering to optimize carboxylases. The improvement of carboxylase activity has a positive influence on CO_2 fixation efficiency. Liu et al. found that the improvement of PCC catalytic efficiency would increase the carbon fixation efficiency of the 3HP cycle by approximately 1.6-fold. It indicates that enhancing the activity of carbon fixing enzymes is a valuable strategy for improving the efficiency of biological carbon fixation. Although progress has been made in optimizing ACC or PCC, some problems remain worthy of attention. For example, it is still unclear what molecular mechanism regulates the substrate selectivity of acyl-CoA carboxylases, and it is difficult to assess the substrate preference of acyl-CoA carboxylases by analyzing their CT components. Therefore, more research on ACC and PCC is needed to meet the needs of biological carbon fixation.

3.2. *Phosphoenolpyruvate carboxylase (PPC)*

PPC (EC 4.1.1.31), an enzyme in the carboxylase family, is a plentiful cytosolic enzyme extensively distributed in plants, cyanobacteria, most archaea, and non-photosynthetic bacteria, but not in fungi, animals, or yeast. PPC catalyzes irreversible β-carboxylation of phosphoenolpyruvate (PEP) to produce oxaloacetate (OAA) and inorganic phosphate using bicarbonate and Mg^{2+} as cofactors. On the one hand, PPC existing in C4 plants or cyanobacteria mediates the initial CO_2 fixation in light conditions during photosynthesis to minimize fixed carbon loss by photorespiration. On the other hand, PPC also contributes to CO_2

fixation through DC/4HB cycle in some chemosynthetic autotrophic archaea, such as *Ignicoccus hospitalis*. PPC carboxylation has received much attention because of its high catalytic rate (enzyme activity of 250 μmol/min/mg), high substrate affinity (K_m of HCO_3^- is 0.1 mM and K_m of PEP is 0.19 mM), and lack of inhibition by O_2 (Tan et al., 2013a). Given the importance of PPC, it is worthwhile to analyze its molecular mechanism to provide clues for developing innovative strategies to convert CO_2. PPC contains four identical subunits with molecular masses ranging from 95 to 110 kDa and is related by crystallographic 222 symmetry, resulting in a molecular symmetry of D2, with vacant holes in the center of its tetramer. The overall reaction of PPC can be dissected into several micro-reversible steps, and a three-step reaction mechanism is the most occurring. In catalysis, PEP is nucleophilically attacked first by bicarbonate, forming carboxy-phosphate and enolate of pyruvate. Then, carboxy-phosphate dissociates into CO_2 and HPO_4^{2-}, and CO_2 nucleophilically attacks the enolate of pyruvate to form the end-product oxaloacetate (**Fig. 4B**).

Various studies have suggested that PPC activity is regulated by intricate allosteric mechanisms. Bacterial PPC can be activated by acetyl-CoA, fructose 1,6-bisphosphate, and long-chain fatty acids and is inhibited by aspartate and L-malate. Masato et al. (Masato and Katsura, 1997) determined that Lys620 in *E. coli* PPC was involved in allosteric inhibition, and a mutant with excellent performance was identified. The PPC mutant (K620S) was almost wholly desensitized to aspartate and L-malate while maintaining nearly the same maximum catalytic activity as wild-type PPC. Plant PPC can be activated by glucose-6-phosphate (G6P) and inhibited by aspartate and L-malate. Kai et al. (Kai et al., 2003) reported that the activity of plant PPC is controlled by specific serine (Ser15) phosphorylation near the N-terminus. S15 phosphorylation activates PPC, making it more sensitive to G6P and desensitizing to aspartate or L-malate. Meanwhile, S15 phosphorylation is catalyzed by a PPC-specific Ser/Thr kinase, and its activity is controlled by its expression level (Kai et al., 2003). Therefore, the host can regulate PPC activity by affecting post-translational control mechanisms such as phosphorylation. Except for allosteric regulation or post-translational modification, PCC activity could also be affected by intracellular substrate concentrations, especially HCO_3^-. Notably, intracellular inorganic carbon concentration is maintained by carbonic anhydrase (CA) and HCO_3^--transport systems. CA can exquisitely and efficiently catalyze CO_2 and HCO_3^- conversion, accelerating CO_2 capture from the extracellular environment into the intracellular system. Chang et al. (Chang et al., 2013; Chang et al., 2014) attempted to integrate CA and PPC to enhance the intracellular inorganic carbon concentration and improve carboxylation efficiency. They found that the conversion value of the CA-PPC system showed a nearly 2.5-fold increase compared to that of PPC due to the increased HCO_3^- availability for PPC due to CA incorporation. Prete et al. (Prete et al., 2016) screened different CA to promote CA-PPC system catalytic efficiency. They found that the CA-PPC system (CA from *Vibrio cholerae*) showed the highest OAA production (3-fold higher than that of PPC). These examples also indicated that the improvement of PPC catalytic performance helps to improve carbon fixation efficiency and enhance the biosynthesis ability of carbon fixation products. They also utilized a similar approach, applying a thermostable PPC from *Thermosynechococcus elongatus* and a thermostable CA from *Sulphurhydrogenibium azorense* to convert PEP and CO_2 to produce OAA, even when exposed to temperatures up to 60 °C. These strategies will improve PPC and broaden its potential applications in biological carbon fixation.

3.3. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)

RuBisCO (EC 4.1.1.39) catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP) with CO_2 to produce two 3-phosphoglycerate (3PG) molecules. RuBisCO determines the carbon assimilation rate of the CBB cycle, which is the main source of organic carbon in the

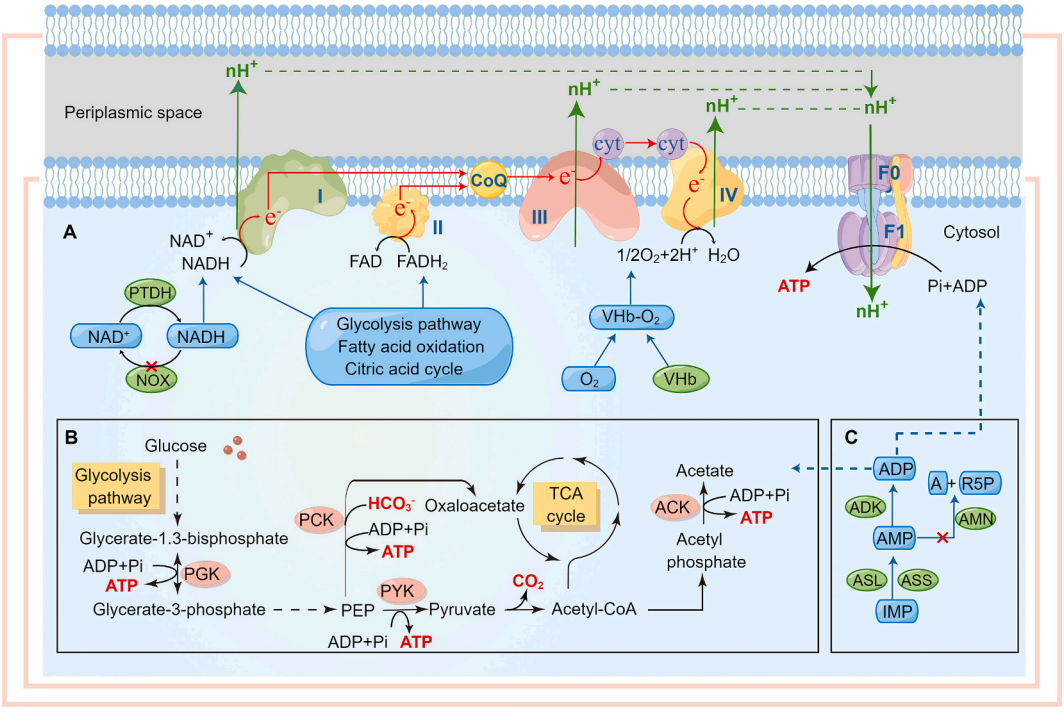


Fig. 4. Strategies for regulating intracellular ATP supply in engineered microorganisms. A: Manipulating oxidative phosphorylation process to regulate ATP concentration. The red lines indicate the flow of electrons and the green lines indicate the translocation of protons. "×" indicate blocking the pathway. PTDH, phosphite dehydrogenase; NOX, NADH oxidase; Cyt, cytochrome *c*; VHb, *Vitreoscilla* hemoglobin; CoQ, ubiquinone; B: Manipulating substrate-level phosphorylation to regulate ATP concentration. PGK, Glycerate-3-phosphate kinase; PYK, pyruvate kinase; PCK, phosphoenolpyruvate carboxykinase; ACK, acetate kinase; C: Manipulating ADP supply process to regulate ATP concentration. ADK, adenylate kinase; ASL, adenylosuccinate lyase; ASS, adenylosuccinate synthetase; AMN, AMP nucleosidase; A, adenine; R5P, ribose 5-phosphate; IMP, inosinate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biosphere. RuBisCO is present in various plants, algae, bacteria, and archaea. According to the phylogenetic analysis of genome sequences, RuBisCO can be classified into four major clades (forms I, II, III, and IV). These four forms are structurally unique, however, the fundamental unit common to all forms is the large subunit dimer. The most widely distributed clade is form I RuBisCO, which comprises eight large subunits (RbcL) and eight small subunits (RbcS), resulting in L_8S_8 stoichiometry. The catalytic sites of RuBisCO are found in large subunits rather than in small subunits. These small subunits provide structural stability and regulate RuBisCO catalytic activity. Concepción Iñiguez et al. (Iñiguez et al., 2020) reported that small subunits are also required for the maximal catalytic activity of RuBisCO but are not strictly necessary for CO_2 fixation. The values for $S_{c/o}$ ($S_{c/o} = (k_{cat\ (CO2)} / K_{m(CO2)}) / (k_{cat\ (O2)} / K_{m\ (O2)})$) of form I RuBisCO (25–240 mol/mol at 25 °C) were higher than those of the other forms (<15 mol/mol at 25 °C). Therefore, form I has a substantially high affinity for CO_2. Forms II and III are composed only of large subunits, resulting in $(L_2)_n$ oligomeric forms. Although the sequence homology among forms I, II, and III was low, they shared highly conserved active sites. Form II RuBisCO has relatively high k_{cat} values (approximately 3–6 s^{-1}) but a significantly lower affinity for CO_2 than form I. Form II RuBisCO is only present in bacteria such as *Rhodospirillum rubrum* or *Thibacillus denitrificans*. Form III RuBisCO is believed to be the most ancient form and is mainly present in archaea, such as *Methanococcus jannaschii* or *Thermococcus kodakaraensis*. Unlike forms I and II, which are involved in the Calvin cycle, form III mainly participates in ribonucleoside assimilation in archaea. It catalyzes the carboxylation of RuBP generated from the ribose moieties of adenosine, guanosine, and uridine to form 3PG. This indicates that form III RuBisCO functions in a metabolic pathway involving nucleoside 5′-monophosphate, and can link the ribose moieties of nucleosides to central carbon metabolism (Riku et al., 2015). Furthermore, form III has the lowest carboxylation efficiency and the lowest $S_{c/o}$ values (0.5–11 mol/mol), which means that form III is highly sensitive to O_2 and has inferior discrimination ability between CO_2 and O_2. Form IV is not involved in the Calvin cycle and cannot catalyze RuBP-dependent CO_2 fixation because of key substitutions of many essential active-site residues. Form IV was first identified in green sulfur phototrophic bacterium *Chlorobium tepidum* and heterotroph *Bacillus subtilis*. It resembles bona fide RuBisCOs (approximately 35% identity at the amino acid level). To distinguish form IV from other RuBisCOs, it is referred to as RuBisCO-like-proteins (RLPs). RLPs are involved in the methionine salvage pathway and sulfur metabolism (thiosulfate oxidation).

Although forms I, II, and III RuBisCO are involved in carbon fixation, they have a slower reaction rate with CO_2. They can only fix 2–10 CO_2 molecules per second in plants, which is several orders of magnitude lower than many common enzymes. Due to the lower turnover, large amounts of RuBisCO are needed to sustain effective net photosynthetic rates. It is estimated that RuBisCO accounts for approximately half of soluble leaf protein, making it one of the most abundant proteins on Earth. During the enzymatic reaction, the third carbon (C3) of RuBP is first deprotonated to generate an unstable enediolate intermediate. Then, the resulting intermediate undergoes an electrophilic attack of CO_2 to produce a branched six-carbon intermediate (carboxy ketone) and is hydrolyzed into two molecules of 3PG (Fig. 3C). This reaction can lead to a net increase in organic carbon. The amino acid residues at the active site were conserved in different RuBisCO forms. Specifically, Lys-175/166/153 participate in the initial deprotonation and final protonation steps. Asp-203/193/181 and Glu-204/194/182 are involved in critical metal ion binding. Lys-201/191/179 are related to the carbamylation of RuBisCO. Arg-279 is necessary for substrate (RuBP) binding. Remarkably, RuBisCO evolves in an atmosphere with a higher CO_2 concentration than the current one, resulting in a low affinity for CO_2 (Vecchi et al., 2020). When O_2 was present in the reaction system, RuBisCO could not differentiate between O_2 and CO_2. O_2 can compete for the same binding sites as CO_2, leading RuBisCO to undergo oxygenase side reactions. In oxygenase reactions, the enediolate intermediate is more likely to react with O_2 than with CO_2 to generate a hydroperoxy derivative intermediate (peroxyketone). The peroxyketone intermediate is further divided into 3PG and 2PG (Fig. 3C). Notably, this reaction does not result in net carbon gain, and 2PG is a toxic compound that can inhibit carbon metabolism and chloroplasts. To recycle 2PG, an energetically wasteful process termed the photorespiratory pathway has evolved for detoxification and carbon recovery. However, it causes the loss of 0.5 CO_2 when scavenging one molecule of 2PG, reducing the net photosynthetic rate. Therefore, the efficiency of CO_2 fixation can be improved by lowering the photorespiration rate. There are many ways to decrease photorespiration. One option is to optimize the carbon enrichment systems (Section 7) and the other is to make a reasonable selection of amino acid residues for the RuBisCO mutation to decrease its oxygen sensitivity.

Tabita et al. found that M295 was placed to interact with the active site in form III RuBisCO using bioinformatic and structural comparison methods. Through site-directed mutagenesis, M295D was identified. Compared to the wild type, there was a 4.8-fold increase in K_O for O_2 from 5 μM to 24 μM, indicating that M295D was more effective in recovering from O_2 exposure. The S363I and S363V mutations had similar functions, and the double mutants (M295D/S363I or M295D/S363V) exhibited an apparent additive effect. When exposed to O_2, double mutants retain much higher activity levels (~90%) than the wild type (~10–15%) (Kreel and Tabita, 2007; Tabita et al., 2008). This indicates that mutations at suitable sites can reduce the O_2 sensitivity of RuBisCO, which is beneficial for improving carbon sequestration efficiency and provides a reference for the functional transformation of RuBisCO. In addition to reducing photorespiration, there are a few ways to increase the catalytic ability of RuBisCO. An alternative strategy for engineering RuBisCO is to use protein-directed evolution based on random mutations and selection. Cai et al. developed an *E. coli*-based activity-directed selection system that can link host cell growth solely to RuBisCO activity. Approximately 15,000 mutants were selected and the best mutant containing two substitutions (E49V and D82G) in the small subunit was identified. This mutant showed an 85% increase in specific carboxylation activity (from 0.91 to 1.68 U/mg) and a 120% increase in the catalytic constant for CO_2 (from 6.7 to 14.6 s^{-1}). Greene et al. (Cai et al., 2014; Greene et al., 2007) used *E.coli* to perform the directed evolution of RuBisCO. After three rounds of random mutagenesis and selection, M295T substitution in large subunit was identified. This evolved mutant showed a 28% increase in carboxylation catalytic efficiency and a 12% increase in the carboxylation turnover rate. It provides an efficient approach to improve the carboxylation activity of RuBisCO and also indicates that the optimization of carbon fixing enzymes helps to improve the efficiency of carbon fixation. Additionally, a functional hybrid of different sources of large and small subunits can be used to optimize RuBisCO. Genkov et al. (Genkov et al., 2010) attempted to assemble small subunits from *spinach*, *Arabidopsis*, and *sunflower* with a large subunit from *Chlamydomonas reinhardtii* to optimize catalysis. Compared to *Chlamydomonas* RuBisCO, hybrid enzymes have an increase of 3–11% in specificity of CO_2/O_2. Furthermore, compared to *spinach*, *Arabidopsis*, and *sunflower,* hybrid enzymes have the highest increases of 65% in carboxylation V_{max}. Although only minor improvements in RuBisCO were observed, the feasibility and effectiveness of hybridizing subunits were demonstrated. Optimizing RuBisCO activation is another option for changing its catalytic performance. In vivo activity of RuBisCO is regulated through complex mechanisms. Multiple studies have shown that RuBisCO can be maintained in an “active state” through the continued action of RuBisCO activase (RCA). RCA is an AAA + protein (ATPases associated with various cellular activities) and a molecular chaperone. It can catalyze RuBisCO activation via ATP-dependent removal of various inhibitory sugar phosphates. Yin et al. (Yin et al., 2010) reported that RCA expression levels affected RuBisCO activity. Concomitantly with the enhanced RCA expression level, the RuBisCO initial activity increased. This result suggests that RuBisCO activation could be a potential target for improving CO_2 fixation

efficiency, and indicates that RCA modulation could also be an attractive experimental goal to improve the carboxylation activity of RuBisCO.

Much effort has been devoted to improving the RuBisCO catalytic efficiency, yet there is still substantial room for improvemen. The current major issues include the fact that mutagenesis always generates activity and/or specificity compromised RuBisCO mutants, as well as the fact that RuBisCO catalysis is challenging to improve by rational design. The partial reasons for limited success in the rational engineering of RuBisCO may be the insufficient understanding of its assembly mechanism, intrinsic structure-function relationships, and the complex cellular machinery required for RuBisCO biogenesis and metabolic maintenance, and so on. Given the importance of RuBisCO in carbon fixation, research on its catalytic mechanism or modification and the proteins associated with its assembly and regulation remains an attractive topic for scientists.

4. ATP supply module

ATP plays an essential role in biosynthesis or metabolism regulation. Maintaining a sufficient ATP supply impacts the CO_2 fixation rate, especially for carbon fixation enzymes in aerobic strains. Based on stoichiometric calculations, Hu et al. present a combinational strategy based on a synergetic CO_2-fixing pathway that combines an ATP-generating pathway with the ATP-consuming shunt. This strategy provides enough ATP to improve the efficiency of CO_2 fixation. Under conditions with sufficient ATP, the fixation efficiency of CO_2 increased by 110% and 870% in *Synechococcus elongatus* and *E. coli*, respectively (Hu et al., 2018). This indicates that a sufficient supply of ATP is important to increase the microbial CO_2-fixing efficiency. ATP can be generated either endogenously or exogenously supplied. The two methods have their characteristics. Exogenous strategies are commonly applied in cell-free systems whose reaction conditions can be modified more quickly than in whole cells. Endogenous strategies are more common and are usually used to optimize microbial cell factories. Strategies for enhancing intracellular ATP production include regulating substrate-level phosphorylation, oxidative phosphorylation, and ADP supply processes.

Under aerobic conditions, most ATP production originates from the oxidative phosphorylation metabolic pathway. This pathway comprises respiratory chain complexes (electron transport chain complexes) and ATP synthase. In general, electrons from reducing equivalents are transferred to the respiratory chain, which drives ATP synthesis through proton gradient-driven ATP synthase (Fig. 4**A**). There are some strategies to enhance cellular ATP supply by regulating the oxidative phosphorylation process. i) Manipulating the ATP synthase. ATP synthases (EC 3.6.1.34) produce ATP from ADP and inorganic phosphate with energy from the transmembrane proton motive force. This enzyme is a rotary enzyme that contains two rotary motors/generators, F0 and F1. Proton-driven F0 and ATP-synthesizing F1 are coupled via elastic torque transmission. When the soluble F1 fraction is free from the ATPase complex, ATP is hydrolyzed into ADP (Li et al., 2020a). Li et al. found that the entire F0F1-ATPase complex overexpression would raise the intracellular ATP/ADP ratio. In the *Saccharopolyspora erythraea* cell factory, compared to the parental strain, the ATP/ADP ratio of the engineered strain with holoenzyme overexpression doubled to 11.4. Conversely, the ratio of a strain with F1-ATPase overexpression was only one-third of that in the original strain. This indicates that manipulating ATP synthase is effective in increasing ATP supply. ii) Regulating O_2 supply. O_2 is the most common terminal electron acceptor in the respiratory chain, and granting sufficient O_2 delivery increases ATP supply. This can be achieved through process control and genetic modification. For the first strategy, aeration with pure O_2, accelerating the agitation speed, and adding O_2 vectors (n-hexane, n-heptane, or n-dodecane) to bioreactors is the most common approach. Zhang et al. (Zhang et al., 2008) added oxygen vectors to the bioproduction of cell factories and found that O_2 supply was improved and carbon metabolism was enhanced. For genetic modification, heterologous expression of *Vitreoscilla* hemoglobin (VHb) is a good choice. It can bind to O_2 at a low concentration of extracellular O_2 and deliver it to the respiratory chain through direct interaction with the terminal respiratory cytochrome, which could facilitate O_2 transfer and improve ATP supply. To increase the concentration of intracellular ATP, Chen et al. (Chen and Tan, 2008) overexpressed the *vhb* gene in engineered *Saccharomyces cerevisiae*. After 24 h, the ATP/ADP ratio of the engineered strain increased by 28% over the control strain. This shows that O_2 supply regulation strategy is valuable for improving ATP supply. iii) Optimizing the viscosity of cell membranes. In oxidative phosphorylation, lipid-soluble ubiquinone diffuses into cell membranes and mediates electron transfer. Man et al. (Man et al., 2020) reported that respiratory metabolism rate is associated with ubiquinone diffusivity. Meanwhile, ubiquinone diffusivity is closely related to the viscosity of cell membranes. The physical properties of biological membranes are determined by their lipid composition. Budin et al. (Budin et al., 2018) reported that increasing the proportion of phospholipid acyl chains with double bonds (unsaturation) decreases the viscosity of the cell membranes effectively. They used FabB (β-ketoacyl-(acyl carrier protein) synthaseI) to produce unsaturated fatty acids for membranes, and its physiological functions were investigated simultaneously. They found that cell membranes viscosity was decreased, and ubiquinone diffusivity and respiratory metabolism rate were increased. This revealed that engineering fatty acid biosynthesis is also an option for optimizing cellular respiration. iv) Enhancing NADH availability. Intracellular NADH is the most important electron donor in oxidative phosphorylation process. Enhancing NADH supply is also an efficient way to control intracellular ATP levels. The major sources of NADH are the glycolysis pathway, fatty acid oxidation, and the citric acid cycle. Overexpression or deletion of NADH-related genes, such as *ackA* (acetate kinase), *aldA* (aldehyde dehydrogenase), *ldh* (lactate dehydrogenase), and *pfl* (pyruvate formate-lyase) could enhance ATP supply. In addition, phosphite dehydrogenase (PTDH) catalyzes the nearly irreversible oxidation of hydrogen phosphonate (phosphite) to phosphate while simultaneously reducing NAD^+ to NADH. To promote ATP synthesis, Chen et al. (Chen and Tan, 2008) expressed the PTDH gene in cell factories, and found that it increased the ATP/ADP ratio by 18%. Furthermore, by using O_2 as an electron acceptor, NADH oxidase (NOX) catalyzes the non-energy-generating oxidation of NADH to NAD^+ to form H_2O, H_2O_2, or superoxide. Man et al. (Man et al., 2016) deleted NOX (encoded by the *noxA* gene) from engineered *Corynebacterium glutamicum* and reported that the NADH level, NADH/NAD^+ ratio, and ATP level increased by 13.6%, 18.2%, and 6.1%, respectively. It also increased ATP-driven metabolites by 13.7%. These results are significant and indicate the feasibility of NADH availability to improve intracellular ATP levels.

Substrate-level phosphorylation is another essential pathway for ATP generation, whether under aerobic or anaerobic conditions (Fig. 4**B**). Glycerate-3-phosphate kinase (PGK) and pyruvate kinase (PYK) produce ATP during glycolysis. Overexpression or co-overexpression of PGK and PYK could increase intracellular ATP levels. Man et al. reported that PYK overexpression in *C. glutamicum* slightly increased intracellular ATP concentrations from 5.54 to 5.59 μmol/g DCW. PGK overexpression could increase ATP concentrations from 5.54 to 5.63 μmol/g DCW. PGK and PYK co-overexpression could increase ATP concentrations from 5.54 to 5.73 μmol/g DCW. Additionally, phosphoenolpyruvate carboxykinase (PCK), which catalyzes the carboxylation of PEP with bicarbonate and ADP to form oxaloacetate and ATP, is another choice for substrate-level phosphorylation. Kim et al. (Kim et al., 2012) overexpressed PCK in *E. coli* BL21(DE3) and found that the ATP concentration in engineered *E. coli* was 1.93-fold higher than the control strain. Acetate kinase (ACK) is an enzyme that mediates the conversion of acetate phosphate to acetate with the concomitant formation of ATP and is crucial in substrate-level phosphorylation. Hunt et al. (Hunt et al., 2010) reported that *Shewanella oneidensis* generates ATP primarily from substrate-level phosphorylation under anaerobic conditions, and ACK is a significant

portion of the substrate-level ATP produced. Mutant strains lacking ACK could not grow anaerobically in minimal medium, whereas wild-type strains could grow normally. This demonstrates the importance of ACK in ATP generation, particularly under ATP-limiting conditions.

On the other hand, ADP is the direct substrate for ATP biosynthesis. Enhancing the ADP supply effectively increases ATP production, whether in oxidative phosphorylation or substrate-level phosphorylation. In ADP synthesis, inosinate (IMP), the initial substrate, is converted into adenosine monophosphate (AMP) by adenylosuccinate synthetase (ASS) and adenylosuccinate lyase (ASL). AMP is then catalyzed by adenylate kinase (ADK) to generate ADP (Fig. 4C). Cai et al. (Cai et al., 2018) overexpressed ADK in *Bacillus licheniformis* to increase the cellular ATP levels. The ATP content of the engineered strain increased to 3.03 μmol/g DCW, which was 27.85% higher than that of the control strain (2.37 μmol/g DCW). Additionally, AMP nucleosidase (AMN) could hydrolyze AMP into adenine (A) and ribose 5-phosphate (R5P). Furthermore, this enzyme is believed to be the main AMP-degrading enzyme. Morrison et al. (Morrison and Shain, 2008) deleted *amn* gene in the wild-type *E. coli* strain and found that it could elevate intracellular ATP levels by >30%. This suggests that improving ADP supply is also an effective strategy to increase ATP supply.

5. The reducing power supply module

NADPH, a critical cofactor in the metabolic network, is integral to the CO_2 fixation pathways (Table 1). However, an insufficient rate of NADPH regeneration often limits the activity of biochemical reactions. To enhance the efficiency of the CO_2 biotransformation process, an alternative approach is to manipulate NADPH regenerating systems. Wang et al. constructed a partial 3HP cycle in cyanobacterium *Synechocystis* sp. PCC 6803 to fix CO_2 and produce 3HP. To improve carbon sequestration efficiency, NAD (P) transhydrogenase gene was introduced into *Synechocystis*. It led to an increase of intracellular NADPH concentration and resulted in a 3.2-fold increase of carbon sequestration capacity (Wang et al., 2016). It suggested that the NADPH supply is also one of the limiting steps for CO_2 fixation and cofactor engineering to increase the availability of NADPH has a positive effect in carbon fixation. There are two significant methods of intracellular NADPH anabolism: i) NADPH-regenerating pathways coupled to the central carbon metabolism. ii) NADPH-regenerating pathways uncoupled to the central carbon metabolism. Method i) is the primary way for NADPH regeneration when glucose is the carbon source. The associated enzymes predominantly include glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), glucose dehydrogenase (GDH; EC 1.1.1.47), isocitrate dehydrogenase (IDH; EC 1.1.1.42), $NADP^+$-dependent malate dehydrogenase (MaeB; EC 1.1.140), and $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GapC; EC 1.2.1.13). Key enzymes in method ii) mainly include NAD^+ kinases, NADH kinases, and transhydrogenases. Compared to method i), method ii) does not affect net catabolic fluxes and is prominent in regenerating and regulating intracellular NADPH levels (Xu et al., 2018).

These three pathways are related to method i). These include the oxidative part of the pentose phosphate pathway (PPP), glycolytic pathway (EMP), and the tricarboxylic acid cycle (TCA). In standard aerobic batch cultivation of *E. coli* on glucose, 35–45% of NADPH generation is contributed by PPP. Fig. 5A demonstrates that G6PDH and 6PGDH are involved in NADPH generation during PPP. Lim et al. (Lim et al., 2002) reported that the NADPH/$NADP^+$ ratio of *E. coli* with 6PGDH overexpression was 2.2, which was 2.0-fold higher than that of the parent strain. Remarkably, G6PDH overexpression resulted in a 6.0-fold increase in NADPH/$NADP^+$ ratio, which was three times more effective than 6PGDH overexpression. This may be attributed to the increased flux of the 6PGDH catalyzing reaction due to G6PDH overexpression. Except for G6PDH and 6PGDH, glucose dehydrogenase (GDH; EC 1.1.1.47) is another way to amplify the PPP (Fig. 5A). GDH can generate NADPH by oxidizing glucose to glucono-1,5-lactone, which can be converted to 6-phosphogluconate in the oxidative part of the PPP. Zhang et al. (Zhang et al., 2011) expressed the GDH gene in *E. coli* BL21 (DE3) and investigated the function of this NADPH regeneration system. They reported that NADPH concentration in the engineered strain with GDH was 4.5 times higher than that in the strain without NADPH regeneration system. The cases mentioned above are significant and fully reveal the effectiveness of G6PDH, 6PGDH, and GDH in supplying

Table 1
Comparison of different CO_2 fixation pathways.

Pathway	Organisms	Substrate	Reaction steps	Products	ATP/one molecular carbon	NAD(*P*)H/one molecular carbon	Carbon fixation enzymes	Reference
CBB	Plant, Algae	CO_2	11	3PG	3	2	RuBisCO	(Liang et al., 2020)
rTCA	*Chlorobiumthio sulfatophilum*	CO_2	9	acetyl-CoA	1	2	2- oxoglutarate synthase and IDH	(Evans et al., 1966)
WL pathway	*Clostridium ljungdahlii*	CO_2	8	acetyl-CoA	0.5	2	FDH, CODH/ACS	(Ljungdahl and Wood, 1969)
3HP cycle	*Chloroflexus aurantiacus*	HCO_3^-	16	pyruvate	1.67	1.67	ACC, PCC	(Strauss and Fuchs, 1993)
3HP/4HB cycle	*Metallosphaera sedula*	HCO_3^-	16	acetyl-CoA	2	2	ACC, PCC	(Berg et al., 2007)
DC/4HB cycle	*Ignicoccus hospitalis*	CO_2, HCO_3^-	14	acetyl-CoA	1.5	2	PFOR, PPC	(Huber et al., 2008)
MCG	*E. coli*	PEP, HCO_3^-	8	acetyl-CoA	3	3	PPC	(Yu et al., 2018)
CBB-MCG	*Synechococcus elongates*	CO_2	–	acetyl-CoA	2.75	2	PPC, RuBisCO	(Liang et al., 2020)
CETCH	in vitro pathway	CO_2	12	glyoxylate	1	1.5	ECRs	(Schwander et al., 2016)
POAP	in vitro pathway	CO_2	4	oxalate	1	0.5	PFOR, PC	(Xiao et al., 2021)
rGly	*E. coli*	CO_2, formate	7	pyruvate	2	3	GCS	(Yishai et al., 2018)
Formolase pathway	in vitro pathway	formaldehyde	5	dihydroxyacetone	1.3	2	NAD-independent formate dehydrogenase	(Poust et al., 2015)
SACA	in vitro pathway	formaldehyde	3	acetyl-CoA	1	1	NAD-independent formate dehydrogenase	(Lu et al., 2019)
ASAP	in vitro pathway	CO_2	11	starch	0.5	2	FLS	(Cai et al., 2021)

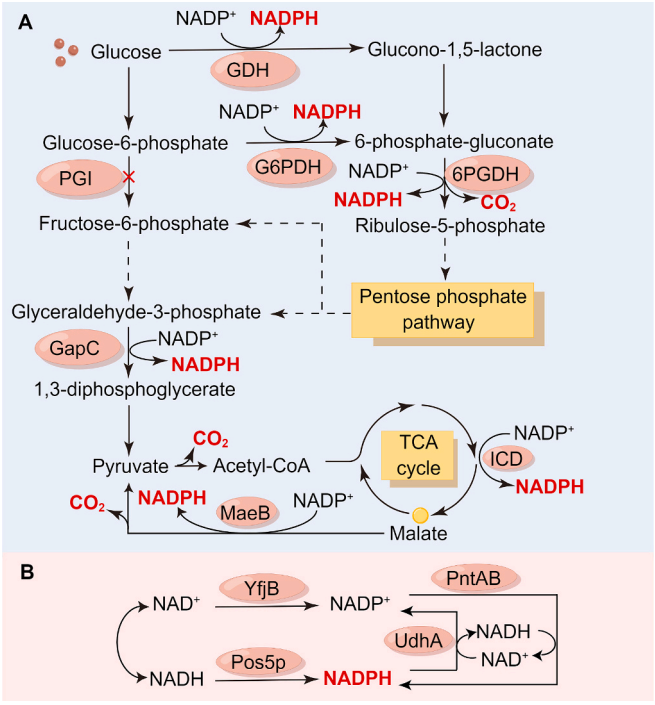


Fig. 5. Strategies for regulating intracellular NADPH supply in engineered microorganisms. A: NADPH-regenerating pathways coupled to the central carbon metabolism. GDH, glucose dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; PGI, phosphoglucose isomerase; GapC, $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase; MaeB, $NADP^+$-dependent malate dehydrogenase; IDH, isocitrate dehydrogenase; B: NADPH-regenerating pathways uncoupled to the central carbon metabolism. YfjB, NAD^+ kinase; Pos5p, NADH kinases; PntAB, membrane-bound transhydrogenase; UdhA, soluble transhydrogenase.

NADPH. Instead of overexpressing the enzymes in the PPP, another alternative is to redirecting the metabolic flux from glycolysis to PPP to increase intracellular NADPH levels. One of the most used methods is to delete the phosphoglucose isomerase (PGI, EC 5.3.1.9). PGI catalyzes a reversible reaction between glucose-6-phosphate and fructose-6-phosphate. The inactivation of PGI could disorganize the competitive EMP and promote the carbon flux to PPP for more NADPH. Chemler et al. (Chemler et al., 2010) evaluated the effect of PGI deletion on NADPH accumulation. The engineered strain *E. coli* BL21 Star™ (DE3) Δ*pgi* showed a 1.3-fold increase in intracellular concentration of NADPH. This result confirms the feasibility of the above method for NADPH regeneration. In EMP, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the transformation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, with NADH or NADPH formation. The common types of GAPDH mainly include NAD^+-dependent GAPDH (GapA; EC 1.2.1.12) and $NADP^+$-dependent GAPDH (GapC; EC 1.2.1.13). Heterologous expression of $NADP^+$-dependent GAPDH would be conducive to enhancing NADPH production. Xu et al. (Xu et al., 2014) replaced GapA with GapC to biosynthesize NADPH instead of NADH in *Corynebacterium glutamicum*. It resulted in a 1.6-fold increase in NADPH/$NADP^+$ ratio, demonstrating the availability of GapC. In the TCA cycle, isocitrate dehydrogenase (IDH; EC 1.1.1.42) and $NADP^+$-dependent malate dehydrogenase (MaeB; EC 1.1.140) are involved in NADPH generation. However, IDH and MaeB overexpression failed to improve NADPH concentration. Conversely, decreasing IDH activity can promote the yield of NADPH-dependent products. These results suggest that neither IDH nor MaeB is a better option for NADPH regeneration than G6PDH, 6PGDH, GDH, and GapC.

NADPH-regenerating pathways uncoupled to the central carbon metabolism are composed of the transhydrogenase system and NAD ($H/^+$) kinase system (Fig. 5B). The transhydrogenase system catalyzes hydride translocation between NAD($H/^+$) and NADP($H/^+$). This system consists mainly of membrane-bound transhydrogenase (PntAB; EC 1.6.1.2) and soluble transhydrogenase (UdhA; EC 1.6.1.1). PntAB catalyzes the transfer of H^+ from NADH to $NADP^+$, producing NADPH. UdhA can transfer H^+ from NADPH to NAD^+ to form NADH. These two enzymes function together to maintain the cellular NADPH/NADH balance. It was reported that 35–45% of NADPH in *E. coli* is produced by PntAB. PntAB overexpression can effectively increase NADPH regeneration. Cui et al. (Cui et al., 2014) overexpressed the PntAB gene in *E. coli,* and the intracellular NADPH concentration showed a 2.3-fold increase. Additionally, researchers have found that the intracellular NADH concentration is higher than that of NADPH. Consequently, the direct phosphorylation of NADH to NADPH has become a potential way to increase NADPH regeneration. Pos5p (EC 2.7.1.86), an NADH kinase, has a high affinity for NADH and could catalyze NADH phosphorylation. Lee et al. (Lee et al., 2013) overexpressed the Pos5p gene from *Saccharomyces cerevisiae* in *E. coli* BL21 (DE3) for efficient regeneration of NADPH, causing a 1.33-fold increase in intracellular NADPH concentration. Furthermore, ATP-dependent NAD^+ kinase catalyzes NAD^+ phosphorylation to $NADP^+$, and it is believed to be the only cell-specific process to produce $NADP^+$. Accordingly, NAD^+ kinase overexpression may increase $NADP^+$ pool size and NADPH abundance. YfjB (NAD^+ kinase; EC 2.7.1.23), which has extremely low specificity for NADH compared to NAD^+, has been used to increase NADPH levels. For example, Zhang et al. (Zhang et al., 2015) demonstrated that the production of NADPH-dependent products increased by 30% with genomic *yfjB* overexpression in *E. coli*. Considering that the combined reaction catalyzed by NAD^+ kinase and transhydrogenase is the same as the Pos5p catalyzed reaction (Fig. 5B), researchers also explored the combinatorial modulation of *yfjB* and *pntAB* genes. Shi et al. (Shi et al., 2013) found that modulating the *pntAB* gene alone increased NADPH-dependent products by 8%, whereas modulating the *yfjB* gene alone had nearly no effect. However, modulating the *pntAB* and *yfjB* genes in combination could increase the NADPH-dependent products by 30%. These results indicat that regulating the transhydrogenase system or NAD ($H/^+$) kinase system could also be recognized as another efficient method for NADPH regeneration and has the potential for use in optimizing CO_2 fixation pathways.

Instead of NADPH, some anaerobic CO_2 fixation pathways, such as the DC/4HB cycle, rTCA cycle, and WL pathway, require ferredoxin as an electron donor. Ferredoxins (Fds) are diminutive proteins (6-13Kda) comprising iron-sulfur (Fe—S) clusters and exhibit a lower redox potential. The Fe—S cluster serves as the prosthetic group of Fd and is located within the hydrophobic module of Fd, imparting Fd with a distinctive brown hue. A single Fd may contain one or more Fe—S clusters. Depending on the number of iron atoms, Fe—S clusters can be categorized into three types: [2Fe—2S], [3Fe—4S], and [4Fe-4S], each of which can be reduced by one electron (Zhang et al., 2022). The iron atoms within the cluster were coordinated by sulfur atoms, forming bonds with the sulfhydryl group of the cysteine residue or inorganic sulfur. In general, the specific type of Fe—S cluster, along with its different redox states and other factors, determines the unique redox potential of the Fe—S cluster. This imparts varying redox potentials to the ferredoxin proteins. For example, under standard conditions (the concentration of substrate or product is 1 mol/L or 10^5 Pa (gas), pH 7.0), the average redox potential values for the [2Fe—2S], [4Fe—4S], and [3Fe-4S] clusters are −300 mV, −400 mV, and between −150 and −100 mV, respectively. Consequently, Fd can dynamically adjust its redox potential by utilizing its Fe—S clusters to accommodate specific redox potential requirements. Within the cellular environment, Fds are >90% reduced allowing them to serve as electron donors in reactions with standard redox potentials as low as or even below −500 mV. An illustrative example of this phenomenon is the natural carbon fixation pathway, in which enzymes such as oxoglutarate synthase or pyruvate synthase (PFOR) catalyze reactions that require extremely low redox

potentials (below −500 mV). To complete the catalytic process, the presence of a reduced Fd is imperative. Currently, Fd with low redox potentials derived from anaerobic organisms, including [4Fe—4S], has been successfully expressed and purified for heterologous use. Furthermore, owing to its significantly low reduction potential, direct reduction of Fd by H_2 or formate is not thermodynamically viable in most circumstances. Consequently, it is imperative to explore the mechanisms that can overcome this thermodynamic obstacle. Electron bifurcation has emerged as a natural solution to this predicament, enabling the production of reduced low-potential Fd from electron donors with relatively high potential, including NADH or molecular H_2 (Nico et al., 2016). Notably, some of these bifurcating hydrogenases have been expressed in *E. coli*, offering insights into achieving substantial intracellular enrichment of reduced potential Fd. In addition, other strategies have also been employed to optimize the production of reduced-state Fd to enhance Fd-mediated metabolic flow. One such process involves using PFOR, which can generate electrons by catalyzing pyruvate breakdown. Agapakis et al. (Agapakis and Silver, 2010) integrated PFOR into metabolic pathway of *E. coli* and incorporated it into the energy metabolism through the hydrogen production circuit of the hydrogenosome. With a reducing potential of −520 mV, PFOR facilitates pyruvate conversion into acetyl-CoA while simultaneously reducing ferredoxin. Subsequently, ferredoxin transfers electrons to hydrogenase, producing H_2 at a potential of −420 mV. The successful expression of PFOR has enabled producing reduced Fd-dependent products at high levels while also serving as a reference for optimizing Fd-dependent carbon fixation enzymes.

6. Energy supply module

Regarding energy utilization, the direct energy sources for biological carbon fixation are ATP and reducing power, which can be acquired from organic substrates, light, chemicals, or electricity. In conventional models of heterotrophic carbon fixation or biosynthesis, organic substrates (e.g., starch or cellulose) serve as the primary energy source. The energy derived from these organic substrates primarily originates from light energy. Based on calculations, the efficiency of the energy conversion process from light to organic substrate to final products was only 0.2%, leaving a significant scope for enhancement (Fig. 6A). This low efficiency is primarily due to inefficient plant photosynthesis and energy loss during the biomass processing and microbial fermentation stages. Autotrophic microorganisms possess the ability to convert CO_2 into biomass using light energy (photoautotrophy) or external inorganic electron donors (chemolithoautotrophy). Unlike conventional energy conversion modes, photosynthetic microorganisms exhibit the potential for higher conversion efficiencies in "light-to-product" ratios, with reported efficiencies ranging from 1%–3% for photoautotrophs. Furthermore, chemoautotrophs demonstrate a "chemicals (e.g., H_2, CO)-to-product" efficiency of up to 7% (Nico et al., 2016). Furthermore, electrical energy can directly or indirectly supply energy to host cells for biological carbon fixation or product synthesis. Electricity generation can be achieved using various renewable sources, including light, wind, tidal, hydro, and geothermal. Currently, the energy efficiency of "light-to-electricity-to-product" is estimated to reach 9%–10%, making it the most efficient known energy conversion process. The above three energy conversion modes provide a reference for achieving an efficient energy supply in the CO_2 biological fixation process. This section presents three distinct energy conversion modes and the primary challenges currently encountered in this field and provides a reference for efficient energy supply for CO_2 fixation.

i) The "light-to-product" energy conversion mode (Fig. 6B). The Calvin cycle in microalgae and the 3HP cycle in *Chloroflexus aurantiacus* use light as an energy source. The rTCA cycle in *Chlorobiumthio sulfatophilum* uses light and sulfur as energy sources. In the 'light-to-product' energy conversion mode, the Calvin cycle, relying on photosynthetic systems, is the most crucial carbon fixation pathway in nature. The average annual solar energy reaching the Earth's surface is approximately 170 W/m^2, but the energy conversion efficiency of photosynthesis is typically very low. In temperate and tropical zones, the conversion efficiency of solar energy for agricultural plants is generally <1%. Furthermore, even in bioreactors, it is only 3% for microalgae, which falls below the theoretical limits. Therefore, it is essential to

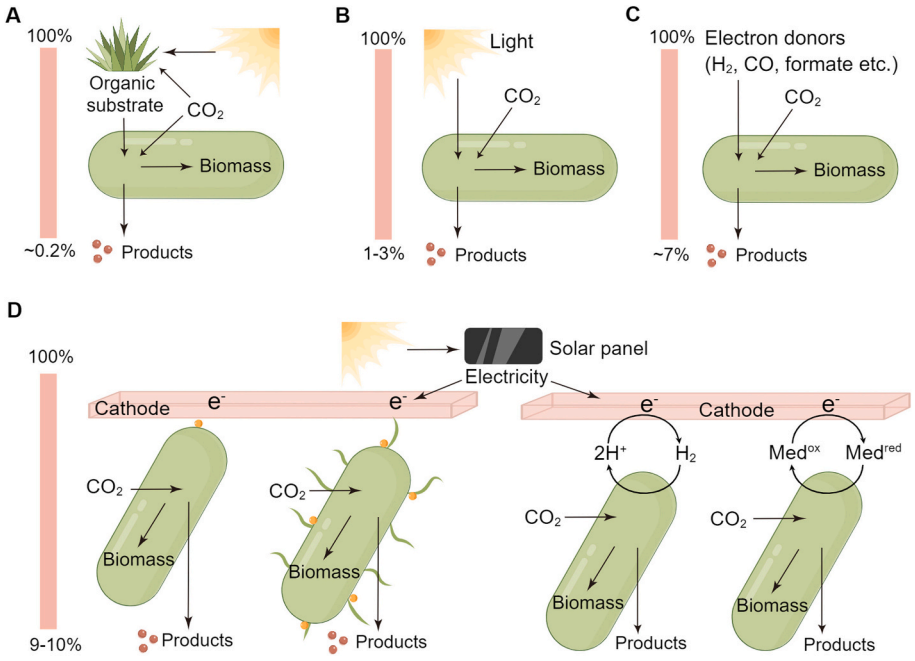


Fig. 6. Different energy supply modes of biological carbon fixation. A: The energy conversion process from light to organic substrate to final products. B: The "light-to-product" energy conversion mode. This process mainly exists in plants, microalgae, photosynthetic bacteria. C: The "chemicals-to-product" energy conversion mode. This process mainly exists in chemolithoautotrophic microorganisms. D: The "light-to-electricity-to-product" energy conversion mode. This process mainly exists in some microbes, such as *Pseudomonas aeruginosa* and *Shewanella oneidensi.* The left side represents electron exchange mediated by cytochrome or conductive pili. The right side represents electron exchange mediated by redox mediators or H_2.

enhance the efficacy of light energy utilization. One potential approach involves employing light of varying wavelengths for illumination. Notably, various forms of photosynthesis exhibit distinct abilities to absorb specific wavelengths of light and photons with varying energy levels. Oxygenic photosynthetic organisms, that employ the CBB cycle, require shorter wavelength light absorption (<700 nm, 176 kJ/mol of photons) (Nico et al., 2016; Venkata Mohan et al., 2016). Conversely, anoxygenic photosynthetic organisms involve longer wavelength light absorption (wavelengths can go beyond 700 nm, 162 kJ/mol of photons). Consequently, if artificial light sources are used to augment the quantity of light, it is imperative that the spectral distribution of the light source closely aligns with the optimal range. Another promising yet arduous strategy for improving sunlight utilization is to expand the range of light wavelengths that photosystems can harness. Photonic engineering is one choice. This method can convert photons between wavelengths and improve solar spectrum utilization. To utilize the ultraviolet portion of sunlight, Yang et al. introduced graphene oxide quantum dots with a 465 nm emission after ultraviolet (380 nm) excitation into the microalgae growth media. As a result, photosynthetic activity was significantly increased and carbon fixation was improved by 20%. It also confirms that increasing energy absorption can help improve the efficiency of carbon sequestration. In addition, exploring alternative photosystems or light-harvesting pigments represents a potential avenue for extending the absorbance wavelength. For instance, bacteriochlorophylls from the photosystems of anoxygenic phototrophs can absorb wavelengths up to 1075 nm (Chen and Blankenship, 2011; Nico et al., 2016). Developing organisms that can produce both bacteriochlorophylls and chlorophylls is one way to increase spectral coverage for light absorption. Recently, Hitchcock et al. successfully demonstrated chlorophyll biosynthesis in the anoxygenic phototroph *Rhodobacter sphaeroides*, although complete integration of chlorophyll and native bacteriochlorophyll pigments has not yet been achieved. Nevertheless, this accomplishment still serves as a proof-of-concept for engineering novel antenna complexes that enhance the spectral coverage of photosynthesis.

Different approaches have been attempted to explore using photogenerated electrons for cell growth and production, including light-activated proton pumps or inorganic-biological hybrid systems. Various photosystems have evolved into natural phototrophic microorganisms in order to harvest light energy. The simplest photosystem is the retinal-pigment-based proton-pumping rhodopsin family. Prokaryotic members of this family include photosensors (sensory rhodopsins), transmembrane proton pumps (bacteriorhodopsins, xanthorohodopsin, and proteorhodopsins), and transmembrane chloride pumps (halorhodopsins). Among them, proteorhodopsins are retinal-containing proteins that catalyze light-activated proton efflux across the cell membrane to generate a proton gradient from light and regenerate ATP. Martinez et al. (Martinez et al., 2007) described a proteorhodopsin-based photosystem that could be functionally expressed in *E. coli* without photopigment. The authors showed that the chemiosmotic potential generated by light-activated proteorhodopsins could activate *E. coli* photophosphorylation via proton translocation. Furthermore, an additional 2.2×10^5 ATP molecules per cell were gained in the above system after 5 min of illumination. This indicates that heterotrophic organisms may utilize the photosystem to convert light into biochemical energy, which can be used for biosynthesis or carbon fixation. Furthermore, the inorganic-biological hybrid system provides another option for the biological utilization of light. Guo et al. (Guo et al., 2018) developed a modular bioinorganic hybrid platform consisting of highly efficient light-harvesting indium phosphide nanoparticles and genetically engineered *Saccharomyces cerevisiae*. In this hybrid system, yeasts could harvest photogenerated electrons from illuminated nanoparticles and use them to regenerate redox cofactors. This process enables decoupling of biosynthesis and cofactor regeneration, facilitating light conversion to metabolites in heterotrophic organisms. This indicates that inorganic-biological hybrid systems could combine the light-harvesting efficiency of inorganic systems with biosynthetic pathways in engineered bacteria, thus promising a sustainable and efficient biochemical synthesis platform (Sakimoto et al., 2017). The above efforts have illustrated the transformative potential of light utilization systems and provided a reference for photoautotrophic synthesis in industrial workhorses. However, these biohybrid systems for photosynthesis are currently in the early stages of development, and their primary challenges include selecting biocompatible light-harvesting devices and seamless integration of biological and non-biological components.

ii) The "chemicals-to-product" energy conversion mode **(**Fig. 6C**).** Inorganic electron donors serve as alternative energy sources for chemolithoautotrophic microorganisms, offering a viable option without light. Common electron donors for chemolithoautotrophic microorganisms include nitrogen oxides, ammonia, hydrogen, sulfur, sulfides, phosphite, ferrous iron, and reduced carbon (CO, methane, methanol, and formate). These compounds can be sustainably derived from waste streams, including industrial combustion gas, or easily regenerated using abundant resources, including light, water, and CO_2. Electron donors can supply reducing power and energy to facilitate CO_2 fixation in chemolithoautotrophic processes. The WL pathway in *Clostridium ljungdahlii* uses hydrogen as an energy source. The DC/4HB cycle in *Ignicoccus hospitalis* and the 3HP/4HB cycle in *Metallosphaera sedula* use hydrogen and sulfur as energy sources. Nevertheless, which of the many microbial electron donors is most suitable for the bioindustry? Claassens et al. (Claassens, 2018) comprehensively evaluated various electron donors, considering their production methods, physicochemical properties, and microbial utilization. Their findings indicated that H_2, CO, and formate are particularly appealing to reducing cellular electron donors. This is due to their ability to be electrochemically produced with high efficiency, low reduction potentials (lower than −400 mV), and high enzyme utilization activities (>10 μmol NAD(*P*)H/min/mg enzymatic system).

Among these electron donors, H_2 is the most studied electron donor for chemoautotrophic growth, and its low reduction potential of $2H^+/H_2$ (–410 mV) allows for the direct reduction of most cellular electron carriers. However, the availability of H_2 is limited. If the energy expenditure associated with the production of H_2 through electrochemistry or photochemistry is calculated and incorporated into the overall energy requirement, it could lead to a 5-fold increase in the energy demand for chemoautotrophic carbon fixation. Consequently, photoautotrophic pathways are more efficient when factoring in the cost of H_2 production. Moreover, utilizing H_2 as an electron carrier in aerobic autotrophic electrosynthesis may raise safety concerns due to its flammability and insolubility. Compared to H_2, CO exhibits a lower redox potential (−520 mV), potentially resulting in a more reduced state of cellular electron carriers and the production of more reduced products, such as ethanol, instead of acetate. When both CO and H_2 are available in a gas mixture, high-energy CO is typically prioritized for utilization (Diender et al., 2015). Like H_2, CO also possesses flammability, explosiveness, volatility, toxicity, and insolubility in water. Therefore, to optimize using H_2 or CO, it is essential to address various engineering challenges related to fermentation, including the design and operation of bioreactors. Additionally, it is imperative to engage in the ongoing screening and development of crucial CO dehydrogenases or oxygen-tolerant hydrogenases. Compared to H_2 or CO, formate has complete solubility, nonflammability, and high redox potential, making it convenient for storage and transportation. Formate also has a lower redox potential (−420 mV), which might make it a more suitable donor. Gleizer et al. integrated synthetic CO_2 assimilation pathways and formate dehydrogenase in *E. coli*, leading to an autotrophic *E. coli* strain. This strain can grow on formate as an energy source and fix CO_2 to produce biomass (Gleizer et al., 2019). It demonstrates the potential value of formate as an electron donor. Besides that, several acetogens and methanogens can also utilize formate as their sole energy source for growth. However, the main obstacle in utilizing formate as an electron donor lies in its toxicity, which could impede many microorganisms

growth at concentrations ranging in the tens of mM. Thereby, screening strains that are tolerant to high concentrations of formate or evolving formate-sensitive organisms to tolerate high concentrations of this donor compound is one strategy to address these challenges.

iii) The "electricity-to-product" energy conversion mode (Fig. 6D). Microbial catalysis in the electroreduction of CO_2 to organic chemicals has emerged as a significant technological advancement, offering a cost-effective alternative for chemosynthesis and serving as a means for carbon capture and fixation. Autotrophic electrosynthesis employs electricity from diverse renewable sources, including light, wind, tidal, hydro, and geothermal, to convert CO_2 into various products within microbial systems. Chen et al. applied a direct current with varying voltage to *N. oceanica* during cultivation. As a result of the applied current, electrons are transferred directly or indirectly into algal cells from the carbon electrode. It was beneficial for the increase of nitrogen/orthophosphate uptake rates and microalgal biomass, and led to a significant increase in carbon fixation efficiency (Cheng et al., 2022). This also indicates that electric energy input is one of the strategies to improve CO_2 fixation efficiency. To date, numerous autotrophic bacteria, including *Clostridium ljungdahlii*, *Moorella thermoacetica*, *Clostridium aceticum*, and *Sporomusa sphaeroides*, have been documented to produce organic chemicals through electrode-driven CO_2 fixation (Zaybak et al., 2013). The electrochemical system operates based on the principle that the simultaneous occurrence of oxidation and reduction reactions at distinct electrodes within a unified system generates an electric current that can be harnessed directly or converted into chemical energy. Regarding energy delivery strategies, microbial electrosynthesis systems can be classified as direct and mediated electron transfer systems. In direct-electron-transferring systems, electron exchange occurs via physical contact between the electrode and microbe, facilitated by outer membrane proteins, such as cytochromes or conductive pili (Fig. 6D). For instance, some *Geobacter* and *Shewanella* strains have developed electronically conducting molecular pili, enabling them to access and utilize solid electron acceptors at greater distances. Additionally, these pili enable microorganisms to utilize an electrode as their sole electron acceptor, even without direct cell contact. Pili are linked to membrane-bound cytochromes, which facilitate electron transfer to the external environment of the cell (Schroder, 2007). Microorganisms frequently thrive in environments lacking soluble electron acceptors or direct access to solid electron acceptors, such as thick biofilms, where O_2 diffusion is limited and cells are not in a direct contact with solid electron acceptors. In this instance, the mediated-electron-transferring systems can facilitate these functions by mediating electron exchange between microbes and electrodes without direct physical contact. The primary mechanism of electron transfer in this system is mediated by redox mediators (electron shuttlers). Microorganisms may either use exogenous redox mediators, including neutral red, AQDS, thionin, methyl viologen, and anthraquinones, or produce redox mediators (flavins and phenazines) via secondary metabolic pathways. To date, some microbes, such as *Pseudomonas aeruginosa* and *Shewanella oneidensis*, have been proven to have the ability to use mediated-electron-transferring systems. In addition to exogenous or endogenous mediators, electron exchange can be initiated by certain reduced metabolites, such as formate or H_2. These can be generated during bioelectrochemical processes, specifically electrochemical reactions occurring at the cathode under electrolysis conditions (Fig. 6D). Suman et al. (Suman et al., 2015) discovered that CO_2 can be converted to acetate through microbial electrosynthesis at H_2 evolving potentials using graphite felt and stainless steel cathode. Significantly, in contrast to direct electron transfer systems, including mediators in this system can increase current, but also introduces selectivity, toxicity, and other concerns.

In different electricity generation modes, solar panels are one of the most promising technologies. Currently, available solar panels demonstrate a light-to-electricity efficiency of ~18%, while emerging advancements have the potential to surpass 40% (Li et al., 2020b). When highly efficient photovoltaic devices are employed, it becomes feasible for the system to convert 9%–10% of solar energy into chemical energy stored in biomass or for carbon fixation (Suman et al., 2015). This energy conversion mode, "light-to-electricity-to-product," demonstrates significantly higher efficiency than biological photosynthesis. It has spurred the development of electricity-driven CO_2 fixation. Further avenues for advancing bio-electrosynthesis from CO_2 include utilizing hybrid production systems that combine autotrophs and heterotrophs or hybrids of autotrophic microorganisms with electrocatalysts or light-harvesting semiconductor materials. Optimizing bioelectrosynthesis and integrating it with established processes shows promise for future development. However, bio-electrosynthesis from CO_2 still faces various operational and technical challenges, including a limited understanding of the electroactivity of biocatalysts, electrode materials, reactor design, or product recovery. In general, bioelectrosynthesis from CO_2 is a promising method for providing energy for CO_2 fixation, offering potential beyond the widely utilized photosynthesis process. Consequently, it is imperative to focus on and address the challenges associated with bio-electrolysis of CO_2 to develop more efficient solutions for CO_2 fixation.

7. Reactor design and technology optimization in carbon fixation

7.1. Reactor design

The electrocatalytic CO_2 reduction reaction (CO_2RR) is a promising way to convert CO_2 and renewable electricity into multi-carbon products (C2+) under mild conditions. During the past few years, considerable improvements in product catalogs, carbon fixation efficiency optimizations, and reactor designs have been made in CO_2RR. The reactor design plays a major role in determining the performance of the electrocatalytic CO_2 reduction reaction process, and different types of reactor designs have been explored for this purpose. In this section, we mainly focus on a description of five main CO_2RR reactor designs, including the comparison of their operating principles as well as their advantages and disadvantages. i) H-shaped reactors. In CO_2RR double-chamber reactors with an H-shaped design are commonly used. The "H-shaped reactor" has an ion exchange membrane that separates two chambers while limiting product crossover and supporting ion conductivity. It is typical for the cathode to reduce CO_2, and the anode to oxidize water to complete the circuit (Fig. 7A). Because of its ease of use and adaptability to a wide range of electrode materials and configurations, "H-shaped reactors" are widely used for carbon fixation. For example, Nevin et al. explored the feasibility of providing electrons to the acetogenic microorganism *Sporomusa ovata* via a graphite electrode to facilitate CO_2 conversion into organic compounds in an "H-shaped reactor". They demonstrated that this device can enable *S. ovata* to use electrons to reduce CO_2 to acetate, and this device also contributes to improving the carbon fixation efficiency of strain (Nevin et al., 2010). However, there are also some drawbacks hindering the capacity of practical applications, such as difficulty in downstream separation and low mass transfer (CO_2) rates. ii) Triple chamber circular reactors. To effectively separate carbon fixation products, a novel modular triple chamber circular reactor is designed. This device contains an anion exchange membrane and a proton exchange membrane, which are used for the concomitant production and separation of carbon fixation products. Three chambers are named anodic chamber, cathodic chamber, and extraction chamber, respectively. The anodic chamber is separated from the cathodic chamber and the extraction chamber by a proton exchange membrane (PEM), which allows protons to migrate from one anodic chamber to another chamber. The cathodic chamber and extraction chamber are separated using an anion exchange membrane (AEM), which facilitates the transfer of acetate ions to extraction chamber, forming acetic acid (carbon fixation products) with protons from anodic chamber (Fig. 7B). Das et al. successfully produced and separated acetic acid using this device, and the carbon fixation rate and

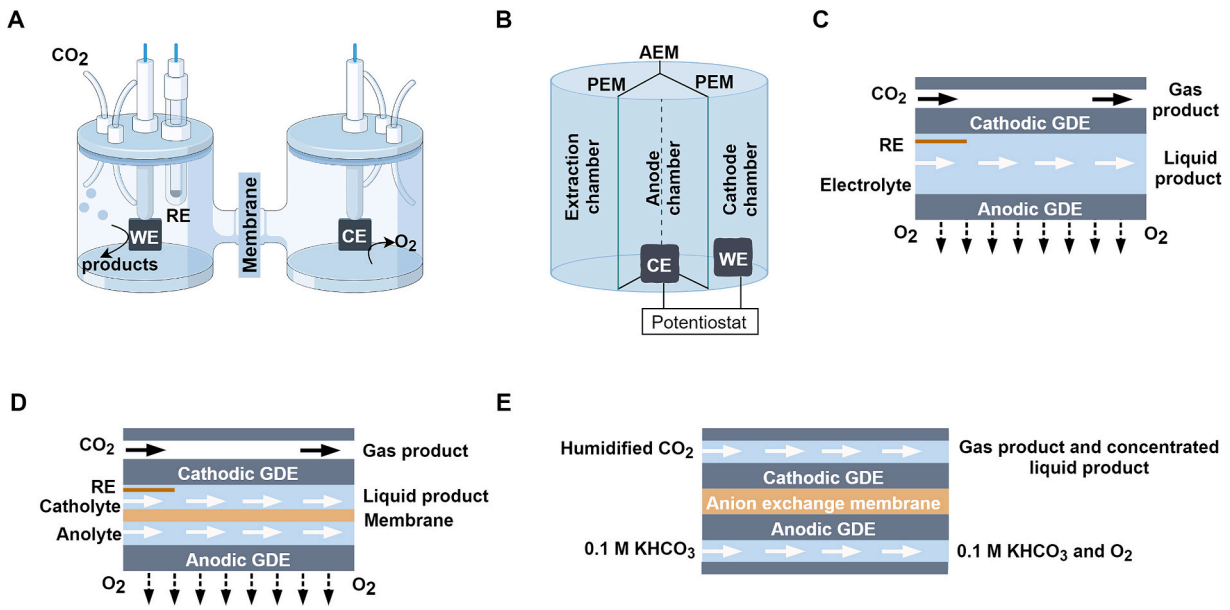


Fig. 7. Schematic diagram of different electrocatalytic CO_2 reduction reaction reactors. A: H-shaped reactor. WE, working electrode; CE, counter electrode; RE, reference electrode. B: Triple chamber circular reactor. AEM, anion exchange membrane; PEM, proton exchange membrane. C: Microfluidic reactor. D: Membrane-based reactor. E: Membrane electrode assembly reactors.

acetic acid production rate are 2–3 times that of conventional methods (Das et al., 2021). Although improvements in carbon sequestration efficiency and rapid separation of products have been achieved, some problems still exist, such as low CO_2 transfer rates and complex structure. iii) Microfluidic reactors. To overcome the mass transport limitations of CO_2, a microfluidic reactor was designed. In this device, a very thin channel (<1 mm) filled with flowing electrolytes separates the two gas diffusion electrodes (GDEs). A chamber was placed behind the cathode GDE so that CO_2 could flow through and reduce, while the anode GDE was left exposed to the atmosphere so that oxygen could escape (Fig. 7C). With a flowing electrolyte, operating conditions can be flexibly tailored to suit varying electrolyte parameters, including exact composition and pH. Compared to the "H-shaped reactor", the involvement of GDEs in this device offers a much thinner diffusion layer, leading to high CO_2 transfer rates. Whipple et al. tested the microfluidic reactor, finding that this device could enable higher current densities, increase carbon fixation efficiency and optimize multi-carbon product production (Whipple et al., 2010). However, several drawbacks should be taken into consideration, such as product crossover (re-oxidation of cathodic products diffused to the anode and reduction of O2 diffused to the cathode), which will decrease the productivity. IV) Membrane-based reactors. To block product crossover in microfluidic reactors, membrane-based reactors have been developed. In membrane-based reactors, an ion exchange membrane is used to separate the anodic and cathodic compartments. Commonly used ion exchange membranes include cation-exchange membranes, anion-exchange membranes, and bipolar membranes (Fig. 7D). Notably, not only neutral electrolyte but also alkaline electrolyte can be used in membrane-based reactors. High alkaline electrolytes allow CO_2 reduction to be carried out more effectively with greater conductivity and improved reaction kinetics than those with lower alkaline electrolytes. Compared to other reactors, a higher current density production of ethylene, ethanol, and acetate from CO_2 has been achieved in alkaline flow cells utilizing membrane-based reactors. It indicates that this device has great prospects in improving carbon fixation efficiency. However, it should be noted that the uptake of CO_2 into alkaline catholyte leads to the formation of carbonates, which may affect the stability of this system. V) Membrane electrode assembly reactors. To solve the carbonate formation problem faced by the membrane-based reactors, membrane electrode assembly reactors (MEA) have been developed. In MEA, the catholyte between the cathode and membrane is removed, and the cathode is in direct contact with the ion exchange membranes in a zero-gap manner (Fig. 7E). The direct contact reduces the chances of contamination of the cathode catalyst by catholyte impurities and reduces ohmic losses to improve energy efficiency. Meanwhile, both the gas and liquid products do not make direct contact with catholyte, which leads to the production of high-concentration liquid products. These features make MEA widely used in CO_2 bio-fixation. Gabardo et al. utilized MEA to fix CO_2 and produce multi-carbon chemicals, achieving ethylene concentrations of 30% at the cathode outlet. The above system can run continuously and stably for >100 h, which is the longest stable ethylene production at high current densities among reported electrocatalytic CO_2 fixation systems (Gabardo et al., 2019). This indicates the enormous potential of membrane electrode assembly reactors in improving carbon sequestration efficiency.

Up to now, reactor design in electrocatalytic CO_2 reduction reactions has promoted the development of microbial electrosynthesis and provided a promising approach to valorizing CO_2 and organic wastes into multi-carbon products. However, further advancements will be required for improving energetic efficiencies, reducing membrane fouling, and optimizing product extraction strategies for ultimate industrialization.

7.2. *Technology optimization in carbon fixation*

The microbial cell factory is at the core of achieving efficient biological carbon fixation. In the construction process of cell factories, the optimization of technology is one of the key factors affecting carbon fixation efficiency. The common technology optimization strategies include metabolic pathway balance technology, protein skeleton technology, high throughput screening technology and gene dynamic regulation technology. i) Metabolic pathway balance technology. Biotransformation of CO_2 is difficult for a single enzyme, and it usually depends on the coordination of multiple enzymes. The caveat here is that the enzyme expression level in a multistep pathway is not simply "the more the better". An excessive metabolic load is not conducive to growth or CO_2 bio-fixation. Metabolic pathway balance is thus necessary for the multistep pathway in CO_2 biotransformation. The common metabolic balance optimization techniques include promoter

optimization, gene copy number optimization, and RBS regulation. Liu et al. achieved metabolic pathway balance by optimizing the copy number of key enzymes in the 3HP cycle. This metabolic balance technique increases carbon fixation efficiency and 3HP production by about four times (Liu et al., 2016). It demonstrates the effectiveness of the metabolic pathway balance technology in improving carbon sequestration efficiency. ii) Protein skeleton technology. The distance between enzymes and substrates in carbon fixation pathways, as well as the spatial position of adjacent enzymes involved in the reaction, usually greatly affect the efficiency of carbon fixation. In order to increase the probability of binding between enzymes and substrates, protein skeleton technology can be used to anchor the enzyme and make the related enzymes gather in a specific area. It can also be used to organize enzyme pathways and metabolite transporters to enhance metabolic flux. Lee et al. have shown that a minor modification to shell protein from the *Citrobacter freundii* could improve its solubility and form filaments in *E. coli*. The filaments can present tractable scaffolds, forming a protein skeleton to tether other proteins and promote metabolic flux (Lee et al., 2017). It provides an instructive example for the construction technology of carbon fixation engineering bacteria and also provides a reference for improving carbon sequestration efficiency. iii) High throughput screening (HTS) technology. Based on protein and cellular levels, HTS uses microplates and automatic operating systems to collect data and quickly complete filtering. It can detect tens of millions of samples at the same time and has the characteristics of being traceable, fast, and accurate. To optimize CO_2 fixation efficiency, some approaches have been developed, such as several random mutation methods and genetic and metabolic engineering. These methods are accompanied by the emergence of a large number of mutants or strains to be screened. Low throughput and slow detection methods limit the effectiveness of conventional screening processes. Therefore, HTS provides a solution for the rapid screening of efficient carbon fixing strains. Scheffen et al. optimized a CO_2 fixing enzyme called glycolyl CoA carboxylase through a combination of mutation design and high throughput microplate screens. This resulted in an improvement of approximately 150% in carbon sequestration efficiency while reducing energy demand (Scheffen et al., 2021). It indicates that HTS is also one of the excellent technical optimization solutions for improving carbon fixation efficiency. IV) Gene dynamic regulation technology. Central to the gene dynamic regulation technology is the realization of the dynamic regulation of metabolic pathways by enabling cells to sense changes in external environmental conditions and turn on or off gene expression at an appropriate time. Common dynamic regulation elements include metabolite-response element, quorum sensing-response element, environmental parameter-response element and protein level regulation element. These strategies are widely used to improve carbon sequestration efficiency. For example, malonyl-CoA is one of the direct carbon fixation products in the 3HP cycle and is often used as a signaling molecule to regulate carbon sequestration efficiency. Xu et al. constructed a bidirectional dynamic regulatory system using transcription factors and promoters responsive to malonyl-CoA to improve carbon sequestration efficiency and yield. In this regulatory system, low concentrations of malonyl-CoA can activate the expression of acetyl-CoA carboxylase while reducing the activity of downstream key enzymes. This can enhance carbon fixation and reduce the consumption of malonyl-CoA, thereby achieving balanced regulation of malonyl-CoA during growth. Compared with unregulated strains, this strategy successfully increased carbon sequestration efficiency by 2.1 times (Yu et al., 2020). In other words, gene dynamic regulation technology can also be a valuable tool for improving carbon fixation efficiency.

8. Progress on carbon enrichment systems

Increasing the substrate concentration is a common strategy to improve the catalytic rate of enzymes. CO_2 or HCO_3^- are key substrates for carbon fixation enzymes, but their content in the cytoplasm is very limited. Therefore, another essential strategy to improve carbon fixation efficiency is to improve the CO_2 or HCO_3^- concentration around carbon fixation enzymes. Xi et al. found that a four-fold increase in inorganic carbon concentration resulted in a 2.1-fold increase in CO_2 fixation efficiency. This indicates the positive effect of carbon enrichment on improving carbon sequestration efficiency. In addition to increasing the supply of CO_2, algae and some autotrophic bacteria have evolved CO_2-concentrating mechanisms (CCM) to internally improve CO_2 concentrations around RuBisCO. CCM is beneficial for overcoming low CO_2 availability caused by slow diffusion of CO_2 in water and reducing photorespiration, thereby improving carbon sequestration efficiency. The CCM comprises two systems: i) an inorganic carbon transport system containing CO_2 pumps and bicarbonate transporters. ii) the carboxysome, a bacterial microcompartment (BMC), which is composed of protein shells, RuBisCO, and carbonic anhydrase (CA). In the CCM system, the HCO_3^- pool in the cytosol first accumulates by two means. External HCO_3^- can be pumped into the cell by bicarbonate transporters of the plasma membrane (BicA, SbtA, HLA3, and BCT1). Alternatively, when the intracellular CO_2 concentration is lower than that of the external medium, CO_2 can enter the cell by simple membrane diffusion and be converted to HCO_3^- by CO_2 hydration enzymes (NDH-I_3 and NDH-I_4) in the thylakoid membrane. In addition, other types of bicarbonate transporters have been identified in *Chlamydomonas*, particularly low carbon inducible transporter A (LCIA) located in the chloroplast envelope and bestrophin-like transporters (BSTs) located in the thylakoid membrane. The accumulated bicarbonate passes through the protein shell and diffuses into the carboxysome. In BMC, HCO_3^- can be converted back to CO_2 by CA, and the carboxysome shell can inhibit CO_2 leakage into the cytosol, which can lead to high levels of CO_2 within the carboxysome to promote RuBisCO CO_2 fixation and reduce photorespiration. It is worth noting that carboxysome shells serve as physical barriers that create a different internal environment from that of the cytosol and control specific metabolite flux in and out of the carboxysome. It permits RuBP and 3PG passage and is selectively permeable to charged HCO_3^-, but it can also preclude O_2 influx and leakage of CO_2 from the carboxysomal lumen (Huang et al., 2022). The selective permeability of the carboxysome shell proteins is presumably related to their unique composition and structure. The carboxysome is a membrane-free organelle with a virus-like shell and encapsulated enzymatic core (RuBisCO and CA). Carboxysomes can be further divided into alpha (Form IA RuBisCO) or beta (Form IB RuBisCO) types according to the type of encapsulated RuBisCO. The alpha type is found in marine cyanobacteria, chemoautotrophs, and purple sulfur bacteria. The beta type is present in freshwater cyanobacteria. CA is another conserved element in alpha- and beta-carboxysomes. The α-carboxysomes contain β-CA, namely CsoSCA. The β-carboxysome CA activity is provided by γ-CA, the role of which is fulfilled by two proteins, CcaA and CcmM. CA and RuBisCO are organized via domain interactions and are surrounded by shell proteins in the carboxysome core. The protein domains that comprise the shells of the two types of carboxysomes are conserved. Shell proteins form polyhedral structures and are mainly composed of hexamers (BMC—H), trimers (BMC-T), and pentamers (BMC—P). During shell formation, BMC-H proteins form hexagonal disks that cover the major surface area of the shell facets. The trimeric pseudo-hexamers of BMC-T proteins appear as hexamers, which comprise a smaller fraction of the shell. During carboxysome assembly, BMC-P proteins are positioned at the vertices of the shell to confine the polyhedral geometry. All shell proteins have a central pore with unique electrostatic properties and are believed to serve as portals for metabolite transport. Liu (Liu, 2022) reported that shell hexamers have positive electrostatic surface potentials, enabling the transport of negatively charged HCO_3^- rather than the uncharged molecules CO_2 and O_2. Despite this, BMC-H proteins have relatively small pores, making it difficult for large metabolites, such as RuBP and 3-PGA, to pass through. Faulkner et al. (Faulkner et al., 2020) proposed a method for large metabolite molecules through BMC-H pores using molecular simulations. They

implied that large-molecule 3PG would experience favorable interactions with shell proteins and induce a conformational change in the shell pore during transit. Cai et al. (Cai et al., 2013) proposed that the pores of the BMC-T proteins may be alternative channels for RuBP or 3-PGA to pass across the shell because trimers have larger central pores than other shell proteins. Therefore, the CCM system exhibites a unique application potential for CO_2 fixation.

The CCM system can increase reaction efficiency by separating a portion of the cellular metabolism. The unique characteristics of CCM provide a reference for optimizing biological carbon fixation. Currently, in the biological fixation of CO_2, the transformation and application of CCM systems depend mainly on three aspects. i) The transfer and integration of carboxysomes or the entire CCM into the eukaryotic metabolic pathways. CCM systems exist mainly in C4 plants, algae, and some autotrophic bacteria but not in major agricultural C3 crops, leading to lower photosynthetic efficiency. It has been estimated that incorporating CCM into C3 crops leads to a 60% increase in carbon fixation. Therefore, increasing attention is being paid to engineering functional CCMs in C3 crops to improve CO_2 fixation and yields. Lin et al. (Lin et al., 2014) replaced the native tobacco gene encoding large RuBisCO subunit with the beta-carboxysomal homolog of the cyanobacterium *Synechococcus elongatus* PCC7942 (Se7942). They demonstrated that Se7942 RuBisCO expression with an internal carboxysomal protein (CcmM) could form macromolecular complexes similar to pro carboxysomes within the chloroplast stroma, mirroring the early step in beta-carboxysome biogenesis in tobacco. This study represents an essential step in improving photosynthesis in C3 plants and provides valuable hosts for building an entire heterogeneous CCM system. Furthermore, Chen et al. (Chen et al., 2023) replaced the gene for large RuBisCO subunits located in the tobacco chloroplast genome with a complete set of genes that encode alpha-carboxysomes of the chemoautotrophic bacterium *H. neapolitanus*. They found that functional alpha-carboxysomes can self-assemble in chloroplasts without any cognate chaperones and support photosynthesis and autotrophic growth of the host at elevated CO_2 and improve carbon sequestration efficiency. This is the first time that functional carboxysomes have been transferred into C3 plants without any cognate assembly and activation factors. This represented a significant step toward installing a fully functional CCM in C3 crops to improve photosynthesis and productivity. Despite this progress, many challenges remain. For example, engineering plants are unable to grow in ambient air, install active bicarbonate transporters or eliminate endogenous chloroplastic CA. Therefore, further research is required to optimize the design of CCM constructs and chloroplast engineering for more efficient CO_2 fixation and photosynthesis. ii) Heterologous reconstruction of the inorganic carbon transport system. Some cell factories can use CO_2 or bicarbonate to produce multi-carbon compounds, including succinic acid, 3-hydroxy propionic acid, and fumaric acid. However, low intracellular inorganic carbon concentrations can limit the activity of critical carbon-fixing enzymes. A common strategy for improving enzyme performance is increasing substrate concentration to increase catalytic rate, especially carbon dioxide concentration. Engineering the cell factory's carbon transport system may be a viable approach to solving this problem. For example, during the fixation of CO_2 to synthesize succinic acid, intracellular CO_2 concentration is closely related to carbon fixation efficiency and succinic acid production. Xiao et al. (Xiao et al., 2017) overexpressed the bicarbonate transporter gene (bicA) from *Synechococcus* sp. strain PCC7002 in succinic acid-producing bacteria. Compared to the control strain, the above engineering strategies resulted in a 59.3% increase in carbon fixation efficiency. This study is a promising starting point, showing the effectiveness of reconstructing inorganic carbon transport systems in carbon-fixing microorganisms and indicating the effectiveness of carbon enrichment systems in improving carbon fixation efficiency. iii) The carboxysomes are engineered to enhance the catalytic efficiency of the embedded enzymes. The unique architecture and semi-permeability of carboxysomes have inspired their rational design and engineering to incorporate enzymes into protein shells for compartmentalization and improved catalytic activity. Li et al. (Li et al., 2020b) expressed six α-carboxysome shell proteins and CsoS2 in *E. coli*, which produced native-like α-carboxysome shells. They also defined the CsoS2 C-terminus as the endogenous encapsulation peptide (EP) that directs external proteins into the shells, making the bioreactor capable of incorporating heterologous enzymes for new catalytic functions. Carboxysomes were transformed into robust and catalytically functional nanoreactors by developing enzyme activation, shell self-assembly, and cargo encapsulation strategies. Compared to hosts that owe free enzymes, the production rate of hosts with nanoreactors increases 4.1-fold. This research demonstrates the potential of carboxysome engineering for catalytic enhancement, enzyme protection, or molecule delivery. This also inspired us to construct functional artificial carboxysomes containing carbon-fixation enzymes, CA, or other enzymes without the original RuBisCO, further enhancing carbon fixation efficiency.

9. Design and construction of synthetic carbon fixation pathways

The utilization of CO_2 is a huge challenge for global sustainable development. Six natural pathways have been discovered for carbon bio-sequestration, however, their low energy efficiency and slow speed limit their industrial application. Recently, several artificial pathways for CO_2 bio-sequestration have been developed with advances in synthetic biology. According to the properties of critical carbon-fixing enzymes, artificial pathways can be divided into carboxylase-mediated artificial carbon fixation pathways and reductase-mediated artificial carbon fixation pathways (Jiang et al., 2020). Unlike natural pathways, synthetic pathways are short, require less energy, and have low crosstalk with natural metabolism. It is possible to improve the efficiency of carbon fixation using more artificial pathways. This section summarizes the two types of carbon fixation pathways and presents the progress in constructing functional components and systems for artificial carbon bio-sequestration.

9.1. Carboxylase-mediated synthetic carbon fixation pathways

9.1.1. The MOG pathway

Except for the WL pathway, five of the six natural carbon fixation pathways are carboxylase-mediated. Among these carboxylases, PPC from the DC/4HB cycle shows the highest catalytic activity and superior affinity for $HCO3^{-}$. Therefore, multiple carboxylase-mediated artificial carbon fixation pathways have been constructed around PPC. Arren et al. (Arren et al., 2010) analyzed the entire set of approximately 5000 metabolic enzymes present in nature. They employed a constraint-based modeling approach to investigate all feasible combinations by utilizing these enzymes as fundamental units. They presented several promising synthetic carbon fixation pathways. A comparison of natural and artificial pathways revealed that all pathways with particular activities have similar cycles and metabolic core structures. They termed this family of core structures the malonyl-CoA-oxaloacetate-glyoxylate (MOG) pathway. Using PPC as the core carbon fixation enzyme, MOG can fix one carbon molecule to generate one glyoxylate molecule. In theory, MOG pathway has better thermodynamic properties, and its carbon sequestration efficiency is 2–3 times higher than that of the CBB cycle. This indicates that the application of MOG has certain advantages in improving carbon fixation efficiency. Regrettably, this model has not been tested in vitro and/or in microbes, but it provides exciting new ideas for subsequent research on constructing artificial carbon fixation pathways.

9.1.2. The MCG pathway

The CBB cycle was the most prevalent CO_2 assimilation mechanism. When its product (3PG) is converted to acetyl-CoA, one fixed carbon atom is lost as CO_2. Yu et al. (Yu et al., 2018) designed a MCG pathway to

reduce carbon loss during acetyl-CoA synthesis using PPC. This pathway can convert one PEP into two acetyl-CoA at the expense of three ATP and three NADH using one PEP and one bicarbonate. If a couple of CBB cycles with the MCG pathway, it allows RuBisCO from photosynthetic cells to assimilate only 1.5 molecule CO_2 to produce one molecule acetyl-CoA with the expenditure of 5.5 molecules ATP and four molecules NAD(*P*)H. Nevertheless, in the native system, the CBB cycle coupled with the native pyruvate dehydrogenase complex requires RuBisCO to assimilate three CO_2 to produce one acetyl-CoA with the expenditure of seven ATP and four NAD(*P*)H. In theory, it is possible to use this coupling pathway to improve energy efficiency and reduce the dependence of photosynthetic organisms on RuBisCO. In addition to carbon fixation, when the MCG pathway is coupled with glycolate dehydrogenase, MCG can convert glycolate to acetyl-CoA with 100% carbon yield, which is favorable for reducing carbon loss during photorespiration. They also demonstrated the feasibility of the MCG pathway in vitro and in *E. coli*. To further evaluate its effectiveness, MCG was implemented in the photosynthetic organism *Synechococcus elongates PCC7942*. Compared to the wild type, the CBB-MCG strain significantly increased the intracellular acetyl-CoA level and maintained good growth status. Isotope labeling was used to investigate the effect of MCG pathway on carbon fixation, finding that the carbon sequestration capacity of engineering bacteria increased by 53.9% compared to the wild type. This indicates that the combination of MCG and CBB is a useful way for improving the carbon fixation efficiency in photosynthetic organisms.

9.1.3. The CETCH cycle

Enoyl-CoA carboxylases/reductases (ECRs) are another excellent choice for carbon fixation, except for PPC. Compared to other carboxylases, ECRs have a wide substrate range, are oxygen-insensitive, and catalyze CO_2 fixation with high efficiency (its specific activity can reach 110 U/mg). However, ECRs are not present in any autotrophic CO_2-fixation pathway. Using ECRs, Schwander et al. (Schwander et al., 2016) designed a completely artificial pathway for continuous carbon fixation in vitro named the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle. This cycle contains 17 enzymes originating from nine different organisms in three domains of life. Under aerobic conditions, CETCH can fix two CO_2 to generate one glyoxylate by consuming two ATP and three NADPH. Compared to other aerobic natural CO_2 fixation processes, CETCH has the fewest reactions and lowest requirement for ATP and NADPH. After multiple optimizations, CETCH can fix CO_2 at a rate of 5 nmol CO_2 min^{-1} mg^{-1} protein, with a carbon sequestration efficiency 93.8% higher than that of the CBB cycle. It indicates that the application of CETCH cycle has potential value in improving carbon fixation efficiency. However, glyoxylate, the final CETCH product, had fewer connections with other major metabolic pathways. Consequently, CETCH may not easily be incorporated into the central metabolic pathways of biological cells. Furthermore, CETCH is based on ATP and NADPH in vitro, which is expensive and unsuitable for practical CO_2 fixation. Accordingly, transplanting the CETCH cycle into heterotrophic hosts may be a logical next step (Schwander et al., 2016). In conclusion, the feasibility of CETCH is a breakthrough, revealing that natural elements can create more efficient artificial carbon fixation pathways.

9.1.4. The POAP cycle

Natural and artificial CO_2 fixation pathways are generally composed of more than eight reactions, which may reduce their efficiency. In light of this, Xiao et al. (Xiao et al., 2021) report the design and experimental demonstration of a minimized synthetic carbon fixation cycle that contains only four reactions. This cycle is named the POAP cycle and comprises pyruvate synthase (PFOR), pyruvate carboxylase (PC), oxaloacetate acetylhydrolase, and acetate-CoA ligase. In the POAP cycle, pyruvate synthase (PFOR) and pyruvate carboxylase perform the carbon fixation function. Theoretically, this cycle can convert two CO_2 to one oxalate at the expense of two ATP and one reducing equivalent in the form of NAD(*P*)H. Under in vitro conditions, POAP cycle can fix CO_2 at a rate of 8 nmol CO_2 min^{-1} mg^{-1} protein, which is 3.1-times higher than the CBB cycle. Compared to the known artificial and natural carbon fixation pathways, the POAP cycle has a higher energy efficiency. It indicates that the POAP cycle has broad prospects for improving carbon sequestration efficiency. However, it is crucial to note that because of the highly oxidized state of oxalate, the POAP cycle requires more ATP and NAD(*P*)H to reduce carbon atoms per valence than the other pathways. Pyruvate synthase is derived from hyperthermophilic anaerobic bacteria, allowing the POAP cycle to fix CO_2 at a high temperature (50 °C) under anaerobic conditions. As a minimized energy-saving CO_2 fixation pathway, the POAP cycle has been demonstrated in in vitro experiments but not in vivo. Therefore, continued efforts must be devoted to oxalate utilization and POAP cycle optimization for their incorporation into the metabolism of microbial cell factories. Briefly, the design of the minimized POAP cycle offers a new avenue for artificial CO_2 biotransformation, providing a model for studying CO_2 fixation in the earliest organisms (Xiao et al., 2021).

9.2. Reductase-mediated synthetic carbon fixation pathways

Except for carboxylase, formate dehydrogenase (FDH) is another carbon-capturing enzyme. FDH catalyzes a reversible reaction between CO_2 and formate. FDH can be classified into two categories: NAD-independent and NAD-dependent. NADH-independent FDH is found in prokaryotes, shows high activity toward CO_2, and is an oxygen-sensitive enzyme. It contains complex redox-active centers that require transition metals (e.g., molybdenum and tungsten) for their activity. NADH-dependent FDH is widely distributed among bacteria, fungi, and plants. This class of FDH is oxygen-insensitive and metal-independent but shows poor activity toward CO_2. In addition to FDH, inorganic methods, such as electrocatalysts, have also been developed to reduce CO_2 to formate using multiple novel chemistries. As Xiao et al. previously reported, when using electrochemical methods to reduce CO_2 to formate, the energy efficiency can exceed 40% (Xiao and Li, 2022).

9.2.1. The reductive glycine (rGly) pathway

The second carbon fixation stage is formate assimilation after CO_2 is reduced to formate. Yishai et al. (Yishai et al., 2018) reported an efficient synthetic route for the aerobic assimilation of formate, the reductive glycine (rGly) pathway. In the rGly pathway, tetrahydrofolate (THF) is ligated to formate and further reduced to 5,10-methylene-THF. Subsequently, 5,10-methylene-THF, CO_2, ammonia, and NADH are catalyzed by the glycine cleavage/synthase system (GCS) to generate glycine. Glycine can be condensed with 5,10-methylene-THF to produce serine. Eventually, serine was reduced to pyruvate for biomass production. To date, the feasibility and effectiveness of the rGly pathway have been validated in *E. coli* using ^{13}C-labeling experiments. Theoretically, this pathway can convert one CO_2 and two formates into one pyruvate at the expense of three NADPH and two ATP. In the rGly pathway, formate can serve as a carbon and energy source (via formate dehydrogenase and oxidative phosphorylation), leading to its high ATP efficiency, which translates to a high efficiency of formate utilization. Consequently, the rGly pathway supports higher biomass and product yields than the natural formate assimilation routes. Beyond this, the rGly pathway is linear, and its metabolic simplicity makes improvements easier than in circular pathways such as the CBB cycle. Furthermore, the rGly pathway has a restricted overlap with endogenous central metabolism and is not sensitive to oxygen, representing a robust functional pathway in both aerobic and anaerobic environments. These advantages make the rGly pathway a highly promising route for implementation in almost any microbe of biotechnological relevance. With these advantages, the rGly pathway has excellent potential for use in virtually all biotechnologically relevant microbes.

Formaldehyde can more easily reach the central metabolism than

formate because of its more active nature. Numerous studies have reported the biotransformation of formate to formaldehyde. Singh et al. (Singh et al., 2018) screened multiple formaldehyde dehydrogenases (FaldDHs) and evaluated their formate reduction capabilities. They identified a novel FaldDH from *Burkholderia multivorans* (BmFaldDH) that exhibited the highest formate reduction activity, catalyzing the formate reduction to formaldehyde. In addition, the ACS-ACDH system can also be used for the bioconversion of formate to formaldehyde. Acetyl-CoA synthase (ACS) catalyzes the ATP-dependent conversion of formate to formyl-CoA, and acetaldehyde dehydrogenase (ACDH) catalyzes the NADH-dependent reduction of formyl-CoA to formaldehyde. After the reduction of formate to formaldehyde, the design of the formaldehyde assimilation pathway is becoming a concern for biological carbon sequestration.

9.2.2. The formolase pathway

Siegela et al. (Siegel et al., 2015) described a computationally designed enzyme, formolase (FLS), that catalyzes the carboligation of three one-carbon formaldehyde molecules into a single three-carbon dihydroxyacetone molecule. By combining FLS with several naturally occurring enzymes (FDH, ACS, ACDH, and dihydroxyacetone kinase), a new carbon fixation pathway was created, the formolase pathway, which can convert CO_2 into the central metabolite dihydroxyacetone phosphate (DHAP). In this pathway, three CO_2 can be converted into one DHAP at the expense of four ATP and six NADH. Additionally, the feasibility of the formolase pathway was confirmed in vitro. This linear and thermodynamically favorable pathway can function under aerobic or anaerobic conditions. Moreover, it is also predicted to use carbon more efficiently and support a biomass yield higher than other pathways (6.5 g cellular dry weight (gCDW)/mol of formate), suggesting its promising applications. However, this pathway also has several limitations. For example, the catalytic efficiency of FLS is relatively low (just 4.7 M^{-1} s^{-1} for the formose reaction), leading to undetectable cell growth with formate as the substrate. However, its side reaction is not easy to control (producing glycolaldehyde), resulting in carbon loss (Poust et al., 2015). Therefore, enzyme engineering and metabolic engineering could be implemented in the formolase pathway for various applications.

9.2.3. The SACA pathway

Lu et al. (Lu et al., 2019) de novo designed a synthetic pathway from formaldehyde to acetyl-CoA, named the synthetic acetyl-CoA (SACA) pathway. In SACA, formaldehyde is first converted into glycolaldehyde (GALD) by glycolaldehyde synthase (GALS). Acetyl-phosphate synthase (ACPS) catalyzes GALD conversion to acetyl-phosphate (AcP). Finally, AcP is converted to acetyl-CoA by phosphate acetyltransferase (PTA). This results in transferring two formaldehyde molecules into one acetyl-CoA molecule through only three steps without consuming ATP or NAD(*P*)H. They demonstrated the feasibility of the SACA pathway in vitro and in vivo using ^{13}C-labeled metabolites. Furthermore, after optimization, the carbon yield reached approximately 50% in vitro. The SACA pathway does not overlap with the known metabolic network, and it is the shortest ATP-independent, carbon-conserving, and oxygen-insensitive pathway for the biosynthesis of acetyl-CoA. However, the high toxicity of formaldehyde to cells and the kinetic bottlenecks of key enzymes result in a trivial contribution of the SACA pathway in vivo. Therefore, improving enzyme activity in this pathway or increasing formaldehyde tolerance would benefit industrial chemicals manufactured from CO_2 using the SACA pathway in the future.

9.2.4. The ASAP pathway

Significant progress has been made in the synthesis using carbon dioxide. Cai et al. reported a chemical-biochemical hybrid pathway for starch synthesis from CO_2 and hydrogen in a cell-free system, named the artificial starch anabolic pathway (ASAP). This pathway contains 11 core reactions and can convert CO_2 into starch at a rate of 22 nmol CO_2 min^{-1} mg^{-1} protein, which is approximately 8.5-fold higher than the starch synthesis in maize and 5.7-fold higher than that of the CETCH cycle (Cai et al., 2021). Compared with other de novo and natural CO_2 fixation pathways, ASAP exhibited the highest carbon sequestration rate. Theoretically, in ASAP, 0.5 molecules of ATP and two molecules of NAD(*P*)H are consumed for each fixed molecule of CO_2. This pathway is based on computational pathway design and is established through four modular assemblies. In the first module (C1 module), CO_2 is chemically hydrogenated to a C1 compound (methanol, formate, or formaldehyde). In the second module (C3 module), the C1 compound is converted to D-glyceraldehyde-3-phosphate by designing and optimizing the key enzyme formolase. In the third module (C6 module), C3 was polymerized into D-glucose-6-phospate using metabolic flux optimization. Eventually, C6 is converted into amylose and amylopectin. According to calculations, the annual starch production of a one m^3 bioreactor using this method is theoretically equivalent to the starch production of corn cultivation on 3333.35 m^2 of land. This pathway allows converting the conventional agricultural starch production method into an industrial workshop production mode, thereby offering a novel technological pathway to synthesize intricate molecules from CO_2 feedstock. However, some challenges remain to be addressed before the potential practical applications of the ASAP pathway can be realized. These include difficulties in extracting the active enzyme and prohibitive production costs. Nevertheless, this approach still provides an important starting point for the industrial biomanufacturing of starch from CO_2.

9.3. The development of artificial autotrophy

In addition, the application of CO_2-fixation pathways also enables hosts to undergo the conversion of heterotrophy to full autotrophy. Some researchers have introduced parts of the CO_2-fixation pathways into heterologous microorganisms for the resource utilization of CO_2. For example, in 2013, Matthew et al. (Matthew et al., 2013) subdivided the 16 stages of the 3HP cycle into four sub-pathways and heterologously expressed each sub-pathway in *E. coli* K12. They found that these four sub-pathways can function to some extent in *E. coli*, providing the possibility of heterotrophic carbon fixation. In 2020, Liu et al. (Liu et al., 2020a) expressed part of the 3HP cycle in *E. coli* BL21(DE3) and constructed a new succinic acid biosynthetic pathway. Isotope labeling experiments demonstrated that this novel route could produce succinic acid from acetyl-CoA with two CO_2 fixation reactions. As compared to traditional methods based on carboxylation of PEP, this new method has a double carbon sequestration efficiency. These studies could transfer CO_2-fixation routes into heterotrophic microorganisms, however, they could not couple CO_2 fixation with cell growth. This was mainly because the carbon flow from the CO_2 fixation route was not directed to the core metabolic network of the hosts (Liu et al., 2022). Biomass carbon is derived from additional carbon sources, rather than CO_2. With the development of synthetic biology, the application research of CO_2-fixation pathways has gradually improved. In 2016, Antonovsky et al. (Antonovsky et al., 2016) employed a rational design to introduce two enzymes associated with the CBB cycle that are absent in *E. coli*. Subsequently, metabolic network transformation and adaptive evolution were implemented, allowing *E. coli* to utilize carbon dioxide to synthesize ~35% of the biomass. These investigations demonstrate the potential for converting heterotrophic organism designs into autotrophs. In 2019, Gassler et al. (Gassler et al., 2020) employed synthetic biology techniques to engineer yeast by adding eight heterologous genes and deleting three native genes, and engineered the peroxisomal methanol-assimilation pathway of *P. pastoris* into a CO_2 fixation pathway resembling the CBB cycle. Using methanol as an energy and electron donor, the resulting strain exhibited continuous growth, relying solely on CO_2 as the carbon source. Simultaneously, Gleizer et al. co-expressed RuBisCO, phosphoribulokinase, and formate dehydrogenase in *E. coli* strain BW25113 Δ*pfkA*Δ*pfkB*Δ*zwf* to fix and reduce CO_2 via the Calvin cycle. After metabolic rewiring and directed evolution, *E. coli* can use

formate as the reducing power and energy source, using CO_2 as the sole carbon source (Gleizer et al., 2019). These studies are significant as they enable the conversion of obligate heterotrophs to full autotrophs by operating the carbon fixation pathways in heterologous hosts. In the process of transitioning from heterotrophic to autotrophic growth, the carbon sequestration ability of microorganisms has changed from zero to some extent. In future research, using the aforementioned organisms as chassis cells to develop cell factories not only has the characteristics of simple genetic operation, but also has the characteristics of product synthesis coupled with biological carbon fixation. As a result, this method of converting heterotrophic to autotrophic also plays an unavoidable role in promoting carbon fixation efficiency. However, in the above process of "artificial autotrophy," oxidizing formate or methanol to supply NADH unavoidably leads to one CO_2 molecule loss, resulting in a net emission of CO_2 under autotrophic conditions. Consequently, several studies must be conducted on "artificial autotrophy" systems, specifically integrating photosynthesis modules or electrocatalytic modules, to mitigate or eliminate CO_2 release. The achievement of sustainable production of multi-carbon compounds will only be feasible if artificial autotrophy can effectively fix CO_2 without any carbon loss.

9.4. Discussion of artificial carbon fixation pathways

Identifying optimal pathways for artificial carbon sequestration depends on several vital features, including minimal enzyme composition, linearity, and disconnection from other metabolic pathways, thermodynamic favorability with a substantial driving force at each stage, and the ability to operate robustly in both aerobic and anaerobic environments. The emergence of Synthetic Biology, particularly the integration of Computer-Aided Design, has facilitated significant advancements in the study of artificial carbon sequestration. In regard to carbon fixation efficiency, the synthetic carbon fixation pathways have surpassed the natural Calvin cycle (2.58 nmol CO_2 min^{-1} mg^{-1} protein). Among these, the ASAP pathway exhibited the highest carbon fixation rate, with a rate of 22 nmol CO_2 min^{-1} mg^{-1} protein. Regarding the total reaction steps, the SACA pathway requires only three steps to synthesize acetyl-CoA using formaldehyde as the substrate. Regarding de novo synthesis of products that use CO_2 as the primary raw material, the POAP cycle requires only four reaction steps, making it the current shortest and simplest carbon fixation pathway. Concerning energy consumption, artificial carbon sequestration pathways tend to exhibit lower energy requirements than natural carbon sequestration pathways. Synthetic carbon fixation pathways, such as the POAP cycle, require only one molecule of ATP and 0.5 molecules of reducing force to fix each molecule of CO_2. Of course, the highly oxidized nature of the resulting products, oxalate in the POAP cycle, contributes to the reduced energy input necessary for these processes (Table 1) (Xiao and Li, 2022). Currently, advancements in the design and modification of artificial carbon fixation components and the assembly and regulation of synthetic carbon fixation pathways, have created carbon fixation systems that surpass natural organisms. Although certain challenges remain, such as the lack of in vivo implementation, the notable benefits of artificial carbon fixation pathways suggest a prospective trajectory for improving CO_2 biological fixation efficiency.

10. Conclusion

Using CO_2 through conversion to organic matter is considered an effective approach. In nature, plants and autotropic microorganisms can fix $>7 \times 10^{16}$ g of inorganic carbon annually via autotrophic carbon fixation pathways. Synthetic biology and metabolic engineering enable heterotrophic organisms to be modified to convert CO_2 into biomass, alcohols, organic acids, and various other chemicals. Presently, the focus of research in the realm of biological carbon fixation revolves around the design and optimization of enzymes, biochemical pathways, and engineered organisms capable of efficient biological carbon fixation. Therefore, this article reviews the thermodynamic properties of various carbon fixation reactions while summarizing the catalytic mechanisms and optimization strategies employed by commonly used carbon-fixing enzymes. The progress achieved in optimizing carbon fixation is also presented, specifically concerning ATP, reduction power, energy supply modules, reactor design, and carbon enrichment system. Additionally, it summarizes and discusses current research on artificial carbon fixation pathways. Despite discovering and developing numerous effective carbon fixation systems, determining the most efficient CO_2 fixation pathway for cell growth and production remains challenge. Several studies suggest that the WL pathway may be suitable for anaerobic CO_2 fixation, particularly in autotrophic electrosynthesis. Similarly, the 3-HP cycle may be the most appropriate pathway for aerobic CO_2 fixation (Liu et al., 2020b). However, no single pathway can be universally ideal for all applications, as its selection depends on various factors, including the cultivation conditions (pH level, energy source), the host (growth rate, feasibility of genetic manipulation, aerobic or anaerobic), and the product (toxicity, energy-deprived or condensed). Furthermore, it is important to note that no single carbon fixation optimization scheme can achieve satisfactory results. Instead, a combination of methods is necessary to enhance the efficiency of biological carbon fixation. Currently, insufficient metabolic efficiency of carbon fixation pathways and inadequate energy supply are unavoidable obstacles to CO_2 fixation. Among the numerous persistent efforts, redesigning carbon fixation pathways using efficient enzymes and exploring efficient bioelectrosynthesis from CO_2 has shown promising potential in improving CO_2 fixation. Integrating diverse carbon fixation modules and innovative energy supply patterns is achievable, offering additional prospects to surpass existing carbon fixation efficiency. Therefore, much work remains to be done to enhance the capacity for biological CO_2 fixation. This effort should primarily focus on the following areas: i) Identifying, designing, and optimizing appropriate enzymes and pathways for carbon fixation. ii) Developing and optimizing efficient carbon transport and concentration systems. iii) Selecting suitable hosts and developing corresponding methods for genetic manipulation. IV) Improving energy capture technology and enhancing energy conversion efficiency. Advances in synthetic biology and biotechnology are anticipated to overcome numerous technical obstacles in the realm of biological carbon fixation, thereby holding promise in meeting the economic demands of carbon fixation.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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