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Review

Opportunities of CO_2-based biorefineries for production of fuels and chemicals

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ABSTRACT

Biorefinery production of fuels and chemicals represents an attractive route for solving current energy crisis, as well as reducing green-house gas (GHG) emissions from ships, planes, and long-haul trucks. The current biorefinery industry is under transition from the use of food (1G, 1st generation), to the use of biomass (2G, 2nd generation). Moreover, the use of atmospheric CO_2 (3G, 3rd generation) has caught increased attention as the possible next-generation biorefinery. Here we discuss how microorganisms can be engineered for CO_2-based biorefineries to produce fuels and chemicals. We start through reviewing different metabolic pathways that can be recruited for CO_2 fixation, followed by different opportunities for CO_2 fixation, either through co-consumption with sugars or used as the sole carbon source. Key challenges and future research directions for advancing 3rd-generation biorefineries are also be discussed.

1. Introduction

The rapid increase in green-house gas (GHG) emissions and reliance on fossil fuels have spurred the production of renewable alternatives, so as to keep the current economy sustainable while reshaping the carbon balance. Currently, 81% of the world's energy consumption and 96 % of organic chemicals rely on fossil fuels [1]. However, the reservation of fossil resources is unlikely to meet human's future requirements. Moreover, the atmospheric CO_2 concentration, which was maintained at 200–280 ppm in the past 4×10^5 years [2], has risen sharply to 421 ppm in the recent 50 years (https://www.co2.earth/). If the current carbon usage pattern continues, the CO_2 level will increase to 500 ppm by 2050 [3], which will affect the ecological system on multiple levels, such as species extinction, increased salinity of ground water, and widespread coastal flooding [4]. The mismatch of time courses for fossil fuel regenerations and release of associated carbon into the atmosphere is at the basis of this problem. Immediate switching from the traditional 'broken cycle' economy to a renewable economy with 'closed cycle' is therefore highly needed [5].

Biofuels can be generated under mild conditions, and thus represents the attractive alternatives for reducing GHG emissions. The versatile nature of microorganisms allows the production of a wide range of fuels and chemicals from a variety of feedstocks, including biomass, biological wastes and a variety of C1 compounds [6]. Selection of appropriate feedstocks generally depends on the price, local availability and environmental impact, whereas these three factors are not always aligned with each other. Costs for feedstocks vary by an order of magnitude, with the lignocellulosic waste being much cheaper than dedicated feedstocks like corn and wheat [7]. Current feedstocks could be grouped into three generations (Fig. 1): food-based generation (1G), lignocellulose-based generation (2G), and C1 based generation (3G).

The first 1G biorefinery plant was built in 1940s by the US Army for fuel-blending [8], and the main driver for expansion of 1G biorefineries was to ensure energy security and reduction of urban air pollution in 1980s [7]. Today 1G biorefineries account for > 95 % of biofuels on the market, including corn starch based biofuels in the US, sugar cane based biofuels in Brazil, as well as maize, wheat and rapeseed based biofuels in Europe and China [9]. Based on the carbon reduction calculation of the US corn ethanol program, even including indirect costs of land uses, corn ethanol reduces ~20–50 % of the CO_2-eq per MJ compared with petroleum [10]. 2G biorefineries, also called lignocellulosic biorefineries, are

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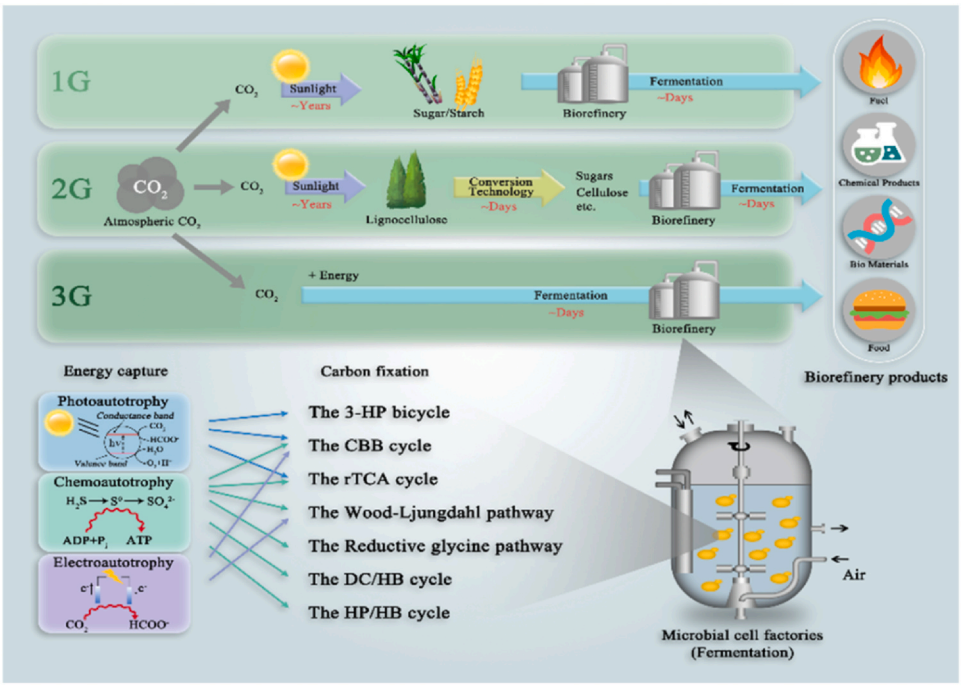

Fig. 1. Scheme and properties of biorefineries. Current biorefineries can be grouped into food-based generation (1G), lignocellulose-based generation (2G), and CO_2 based generation (3G). 1 G biorefineries are mainly based on consolidated bioprocessing of seeds, grains and sugars. 2G biorefineries mainly use feedstocks derived from fast growing trees and perennial grasses. 1G & 2G biorefineries sometime evoke concerns about the competition of land and water, as well as the energy-consuming raw material processing. On the other hand, 3G biorefineries tend to circulate renewable resources and CO_2 in a closed-loop.

mainly using feedstocks derived from fast growing trees and perennial grasses [7]. 2G feedstock with its abundance and yearlong availability, has attracted much attention. Currently we are in the transition from 1G biorefinery to 2G biorefinery, with the first lignocellulosic biofuel plants implemented in 2004 [11]. In 2008, the Chinese government has initiated programs to grow non-grain biofuel feedstocks like sweet sorghum and cassava on saline, barren and waste lands [12]. Many different companies, including DuPont, Abengoa and POET, have also started to join the field using enzymatic processed lignocelluloses to produce ethanol [13]. However, except for waste lignocellulosic biomass, 1G and 2G feedstocks are continuously concerned, due to their requirements for arable land and fresh water, reduction in biodiversity, and release of N_2O from fertilized soils [7].

On the other hand, 3 G biorefineries aim to utilize renewable energies to convert C1 compounds, including methane, methanol, syngas, formic acid, and ultimately atmospheric CO_2, to fuels and chemicals [5]. Here we will focus on CO_2-based biorefineries, and for other biorefineries please refer to recent literatures [14,15]. Proof of concept studies of CO_2 biorefineries has been made in cyanobacteria for production of commodity chemicals as well as short and medium chain alcohols [16]. Recent works also illustrated that two widely applied microbial cell factories were converted from heterotrophs to simi-autotrophs, and eventually to full autotrophs capable of growth on CO_2 [17]. However, because of the low energy capture efficiencies, sub-optimal CO_2 capture and conversion rates, these processes are currently not economically viable. Here we review how microorganisms have been engineered for CO_2 fixation for production of fuels and chemicals, including different CO_2 utilization models and fixation pathways, and end with perspectives on future research directions.

2. CO_2 utilization models

This section may be divided by subheadings. Assimilation of the very stable and low energy configuration of CO_2 into cellular carbon demands four reducing equivalents and a lot of energy [18]. This demand can be obtained through co-consumption of CO_2 and sugars, or other energy sources such as light, chemicals and electricity, that will require cleverly designed catalysts and a global rearrangement of carbon fluxes.

2.1. Co-consumption of CO_2 with sugars

1 G and 2 G biorefineries have focused on heterotrophic fermentations to convert plant biomass into the desired product, typically ethanol; however, the carbon yields in these processes are far from optimal. For example, one third of the carbon is lost to CO_2 at pyruvate dehydrogenase/decarboxylase and one sixth of the carbon is lost at 6-phosphogluconate dehydrogenase. Therefore, a more carbon-efficient strategy is desirable, and could be achieved with co-consumption of 1G and 2G feedstocks with CO_2. More importantly, this could also be considered as the proof-of-concept stage for evaluating pathways and enzymes that could be used when CO_2 serves as the sole carbon source.

Regarding co-consumption of CO_2 with sugars, there are mainly two routes developed, either through introduction of efficient heterotrophic pathways into naturally occurring autotrophic organisms such as algae and cyanobacteria, or through expression of CO_2 fixation pathways into well studied cell factories such as *Escherichia coli* and *Saccharomyces cerevisiae*. For example, Lee *et al.* reported that heterologous expression of xylose isomerase and xylulokinase in *Synechocystis sp.* enhanced keto acid production, with half of the carbon derived from xylose and the other half from CO_2 [19]. Moreover, Gleizer *et al.* reported the construction of autotrophic *E. coli* via the Calvin-Benson-Bassham (CBB) cycle, with the biomass synthesis solely derived from CO_2 and the energy harvesting solely from formate [20]. Hu *et al.* integrated a synthetic CO_2 fixation pathway (half-Wood–Ljungdahl-formolase, HWLS) in *E. coli*, together with the self-assembled nanoparticles to generate light-driven reducing power, and enhanced malate and butyrate production approaching to the theoretical yield [21]. Meanwhile, reactions in the CBB cycle have also been employed in *S. cerevisiae* to enhance the production of free fatty acids [22].

Besides the introduction of carbon fixation pathways to recruit CO_2 released during the biorefinery process, another approach is to introduce carbon conservation pathways to bypass CO_2 releasing reactions. For example, two carbon conservation routes between glucose and acetyl-CoA were reported, including the reverse glyoxylate shunt (rGS) [23] and the non-oxidative glycolysis (NOG) [24], as shown in Fig. 2. Qin *et al.* blocked yeast glycolysis and integrated phosphoketolase from *Leuconostoc mesenteroides* and phosphotransacetylase from *Clostridium kluyveri,* and improved the production of free fatty acids in

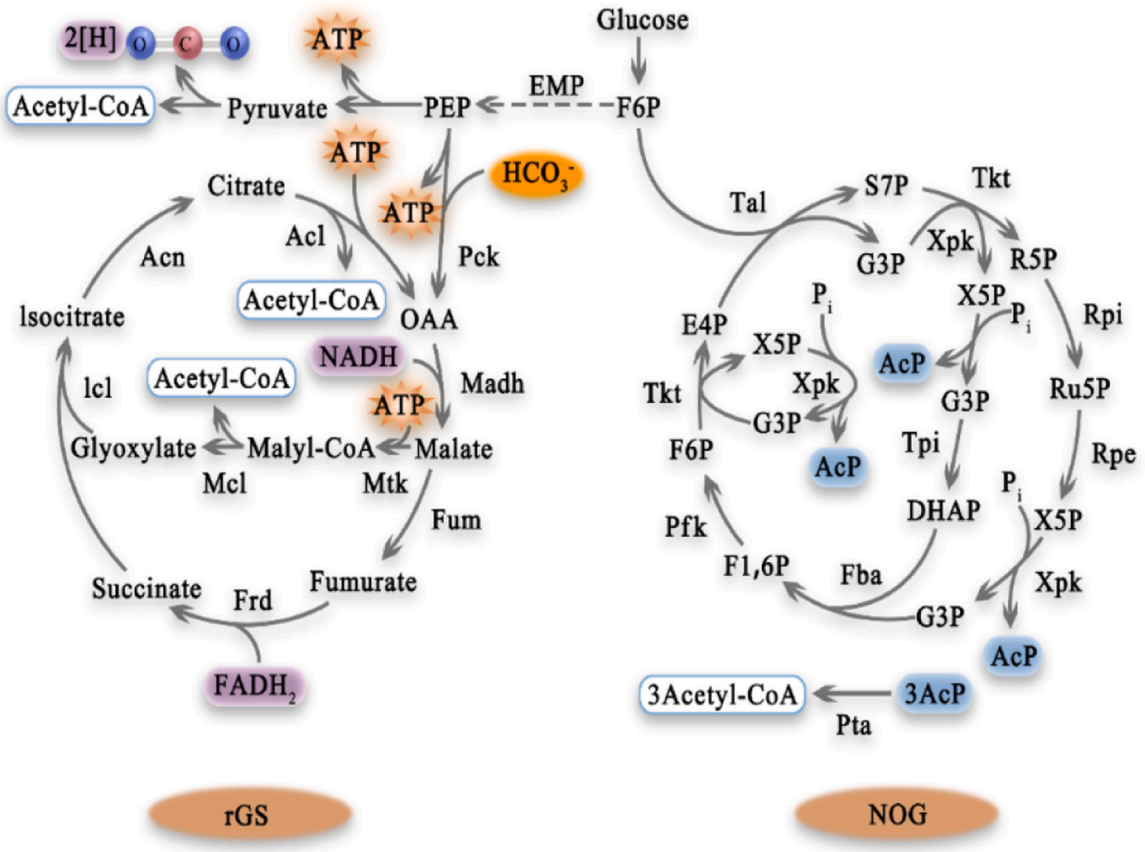

Fig. 2. Illustrative examples of CO_2 conservation pathways. The reverse glyoxylate shunt (rGS) and the non-oxidative glycolysis (NOG) as potential routes to conserve carbon for both microbial growth and production.

S. cerevisiae [25]. Similarly, Bruinsma *et al.* integrated phosphoketolase and phosphotransacetylase into *Pseudomonas putida*, and increased the production of malonyl-CoA and mevalerate using glycerol or xylose as the substrate, respectively [26]. However, it is important to note that a significant amount of CO_2 still releases into the environment through these processes. The lost carbon can be recaptured by *Clostridium* through the Wood–Ljungdahl pathway, to produce formic acid and acetic acid, which could be fed back to the above-mentioned heterotrophic cell factories as additional carbon sources. To be more specific, the stoichiometry converting glucose to FFAs, for example, stearic acid, is $7.14\,C_6H_{12}O_6 + 7.84\,O_2 + 18\ NAD^+ \rightarrow C_{18}H_{36}O_2 + 18\ NADH + 24.84\ CO_2 + 6.84\,H_2O$, whereas the stoichiometry for FFAs productions through co-utilization of glucose and acetic acid is $9\,C_2H_4O_2 + 3.29\,C_6H_{12}O_6 + 9.79\,O_2 + 3.9\ NAD^+ = C_{18}H_{36}O_2 + 3.9\ NADH + 19.74\ CO_2 + 15.84\,H_2O$. Furthermore, if we consider all the acetic acid is from CO_2 fixation through the Wood-Ljungdahl pathway in *Clostridium*, the overall stoichiometry is $3.29\,C_6H_{12}O_6 + 9.79\,O_2 + 72\,H^+ + 72e^- + 3.9\ NAD^+ = C_{18}H_{36}O_2 + 3.9\ NADH + 1.74\ CO_2 + 33.84\,H_2O$. Moreover, the overall amount of CO_2 released will be further reduced if we take into account of the CO_2 conservation reactions.

In addition to reforming microorganisms to achieve biological carbon sequestration, cell-free systems for carbon sequestration are also gaining popularity as they have the potential to increase the rate of carbon sequestration towards physical and chemical limits. Luo *et al.* constructed a cell-free CO_2 fixation system comprising a synthetic reductive glyoxylate and pyruvate synthesis (rGPS) cycle and the malyl-CoA-glycerate (MCG) pathway [27]. A real-time opto-sensing module was designed to control cofactor regeneration. In the future, *in vitro* carbon sequestration systems could potentially be inserted into a suitable microorganism. In order to achieve this goal, it is necessary to standardize and engineer *in vitro* carbon fixation pathways, which is the vision and direction of synthetic biology.

2.2. 3G biorefineries

Depending on the energy assimilation techniques, biological systems in 3G biorefineries can be divided into phototrophs, chemoautotrophs and microbes that could utilize electricity. The progress of CO_2 assimilation by engineered microorganisms in recent years is shown in Table 1.

Photosynthesis utilizes photon energy to convert H_2O and CO_2 into organic compounds. Among different photosynthetic organisms, microalgae and cyanbacteria have been widely applied to assimilate CO_2 to produce fuels and chemicals, such as oleochemicals [28] and aromatics [29]. Taking microalgae as an example, its annual production level is reaching 5000 ton of dry algal biomass, which can be further converted to fuels and chemicals [30]. Attractive photoautorophs include but are not limited to photoautotrophs *Scenedesmus obliquus* that has already been commercialized [31] and *Rhodobacter sphaerodes* that could also fix nitrogen [32]. Photosynthesis plays important roles in energy generation and carbon fixation, and converts unlimited resources (sunlight, water and CO_2) to desired bioproducts. However, photosynthesis requires large light-exposure surfaces and is limited by energy capture efficiencies [9]. Thus, closed cultures are very costly, whereas open-pond cultivations may suffer from contaminations and unstable cultivation controls.

Chemoautotrophs fix CO_2 obtaining energies from chemical electron donors, such as hydrogen, ammonia, phosphite, sulfur (S, H_2S) and metal irons [33]. Most of these energies can be obtained from waste streams and regenerated by light and electricity. It has been suggested that H_2, CO and formate are more attractive compared with other

Table 1
Recent developments in the assimilation of CO_2 producing chemicals.

Strain	Substrate	Pathway	Products	Yield	Reference
Pichia pastoris	CO_2	CBB	Itaconic acid	2 g/L	[50]
Citrobacter BD11	CO_2	rTCA	Succinic acid	7.5 g/L/d	[39]
Cupriavidus necator	Formate and CO_2	CBB cycle	PHAS	70% of the totalbiomass	[85]
Acetobacterium woodii	CO_2/H_2	Wood–Ljungdahl pathway	Acetate	51 g/L	[86]
Pyrococcus furiosus	CO_2	HP/HB	3-hydroxypropionic acid	0.02 g/L	[79]
Escherichia coli	Formate and CO_2	Wood–Ljungdahl pathway	Glycine and Serine	-	[87]

electron donors, because of their low reduction potentials that could directly reduce cellular electron carriers [34]. Attractive chemoautotrophs include *Ralstonia eutropha* and *Clostridium acetogens*. *R. eutropha* is able to oxidize H_2 or formate to store carbon in the form of polyhydroxyalkanoates (PHAs) in an amount up to 70 % of the dry weight [35], or to produce desired chemicals, including diesel-range methyl ketones [36], and hydrocarbons [35]. Clostridium species, on the other hand, could utilize most biomass-derived carbohydrates as well as waste streams and C1 compounds to produce a wide range of products, including butanol, 2-oxobutyrate and 3-butanediol [37,38]. Interesting, a new species of *Citrobacter* was found through genome sequencing, which could convert CO_2 to succinic acid with a production rate of 7.5 g/L/d [39].

Recently, another attractive route has been developed using electricity to provide energy and reducing equivalents to microbial systems for more efficient CO_2 assimilation and utilization. This may at the same time enable storage of electrically generated energy in terms of chemicals with high-energy density. Various value-added products can be produced from this route, for example, acetic acid and bioalcohols [40]. Depending on the energy assimilation techniques, it can be divided into electron transferring systems that can directly consume electrons in a low driving voltage environment, and energy carriers transferring systems in which electricity is utilized to generate electron carriers and these electron carriers are used to assimilate CO_2 and support cell growth [41]. Electron transferring systems could support electricity-to-product at high energy efficiencies (up to 90 %) [42], whereas may suffer with low current density, thus low productivities [34]. Generally, anaerobic conditions are more electrosynthesis favorable, since without oxygen there are less electrode corrosion, fewer safety concerns (especially when using H_2 or CO as electron carriers) and higher energy conversion efficiency compared with aerobic conditions [43].

3. CO_2 fixation pathways

Current identified CO_2 fixation pathways can be grouped into the following classes, as shown in Fig. 3. Unique features of each class, as well as advantages and limitation factors of each CO_2 fixation pathway are discussed here. For detailed chemistry of each pathway, please refer to recent reviews [6,44].

(i) The CBB cycle fixes CO_2 through the pentose phosphate pathway [45]. The CBB cycle is the most widely used carbon fixation pathway in autotrophic organisms [46], with its key enzyme RuBisCO being notoriously inefficient [47]. Although a series of studies have enhanced current understanding for the molecular basis of RuBisCO [48], only few active RuBisCO enzymes were reported with limited improvements. Antonovsky *et al.* introduced the CBB cycle in *E. coli* and developed a screening system based on ribulose phosphate kinase (Prk) and ribulose 1, 5-diphosphate (RuBP) to achieve directed evolution of RubisCo [49]. Baumschabl *et al.* expressed lactic acid and iconic acid synthesis genes in autotrophic *Komagataella phaffii*, and enabled its production of organic acids through the CBB cycle using only CO_2 as the carbon source [50]. Moreover, CO_2 concentrating mechanisms have been integrated in *E. coli* and *Pichia pastoris* to increase local CO_2 concentrations near RuBisCO [17,20].

(ii) The Wood-Ljungdahl pathway [51] and the reductive glycine pathway [52] both employ the ATP-free CO_2 reduction reactions, and are more energy efficient [53]. The downside of these pathways is that they both require complex reaction assistants, such as metals, cofactors and chaperones [54]. For the Wood-Ljungdahl pathway, different hosts differ in the use of reducing powers, energy requirements and coenzymes [55]. For example, in the methyl branch of this pathway, methanogens use chemiosmotical energy to reduce ferredoxin rather than ATP, whereas acetogens use NADPH rather than ferredoxin, with one additional ATP equivalent required [18,56]. Key enzymes of the Wood-Ljungdahl pathway are formylmethanofuran dehydrogenase/ formate dehydrogenase/ CO dehydrogenase [57]. Among these enzymes, CO dehydrogenase is an oxygen-sensitive enzyme, and thus the Wood-Ljungdahl pathway could only work under strictly anaerobic conditions. Recently, a new CO dehydrogenase from *Desulfovibrio vulgaris* was found to tolerate oxygen [58]. On the other hand, the reductive glycine pathway that shares four reactions with the Wood-Ljungdahl pathway (Fig. 3) was originally proposed to be a synthetic pathway for CO_2 fixation [59], and identified as an native pathway in the sulfate-reducing bacterium *Desulfovibrio desulfuricans* [60]. The glycine cleavage/ synthase system (GCS) that catalyzes the generation of glycine from 5,10-methylene-THF and CO_2 is fully reversible, and thus limits the flux to support autotrophic cell growth [61]. The implementation of the reductive glycine pathway on methanol/ formate and CO_2 for production of the essential metabolites glycine and serine has already been demonstrated in *E. coli* [62], *S. cerevisiae* [63], and *P. putida* [64].

(iii) The reductive TCA cycle [65] and the dicarboxylate/ 4-hydroxybutyrate (DC/HB) cycle [66] fix CO_2 using acetyl-CoA/succinyl-CoA cycles. Critical steps in the reductive TCA cycle are catalyzed by ATP-citrate lyase and the oxygen sensitive 2-ketoglutarate synthase [46,67]. It was generally accepted that citrate synthase drives an irreversible reaction to form citrate from oxaloacetate and acetyl-CoA, and thus cannot be used for autotrophic growth [68]. Interestingly, two recent studies demonstrated that citrate synthases that could catalyze both directions naturally exist [68]. Regarding the other key enzyme in the reductive TCA cycle, 2-ketoglutarate synthase catalyzes the carboxylation of succinyl-CoA using ferredoxin as the reducing power. The structure of this enzyme has been reported to reveal the molecular basis of substrate specificity, catalytic bias, and reaction directionality [69], providing information to future engineering for more efficient carbon fixation. On the other hand, the DC/HB cycle assimilates both CO_2 and bicarbonate through phosphoenolpyruvate carboxylase. The key enzyme in this pathway is the FAD-containing 4-hydroxybutyryl-CoA dehydratase that catalyzes 4-hydroxybutyryl-CoA to crotonyl-CoA [67]. Recently, the enzyme was investigated in more details with crystal structure and site-directed mutagenesis experiments, characterizing its molecular basis of oxygen tolerance [70]. Currently, the DC/HB cycle has been identified both in anaerobes, such as *Desulfurococcales* [66], and facultative aerobes, such as *Pyrolobus* [71].

(iv) The 3-Hydroxypropionate (3-HPA, Fuchs-Holo) bicycle [72] and the 3-hydroxypropionate/ 4-hydroxybutyrate (HP/HB) cycle [73]

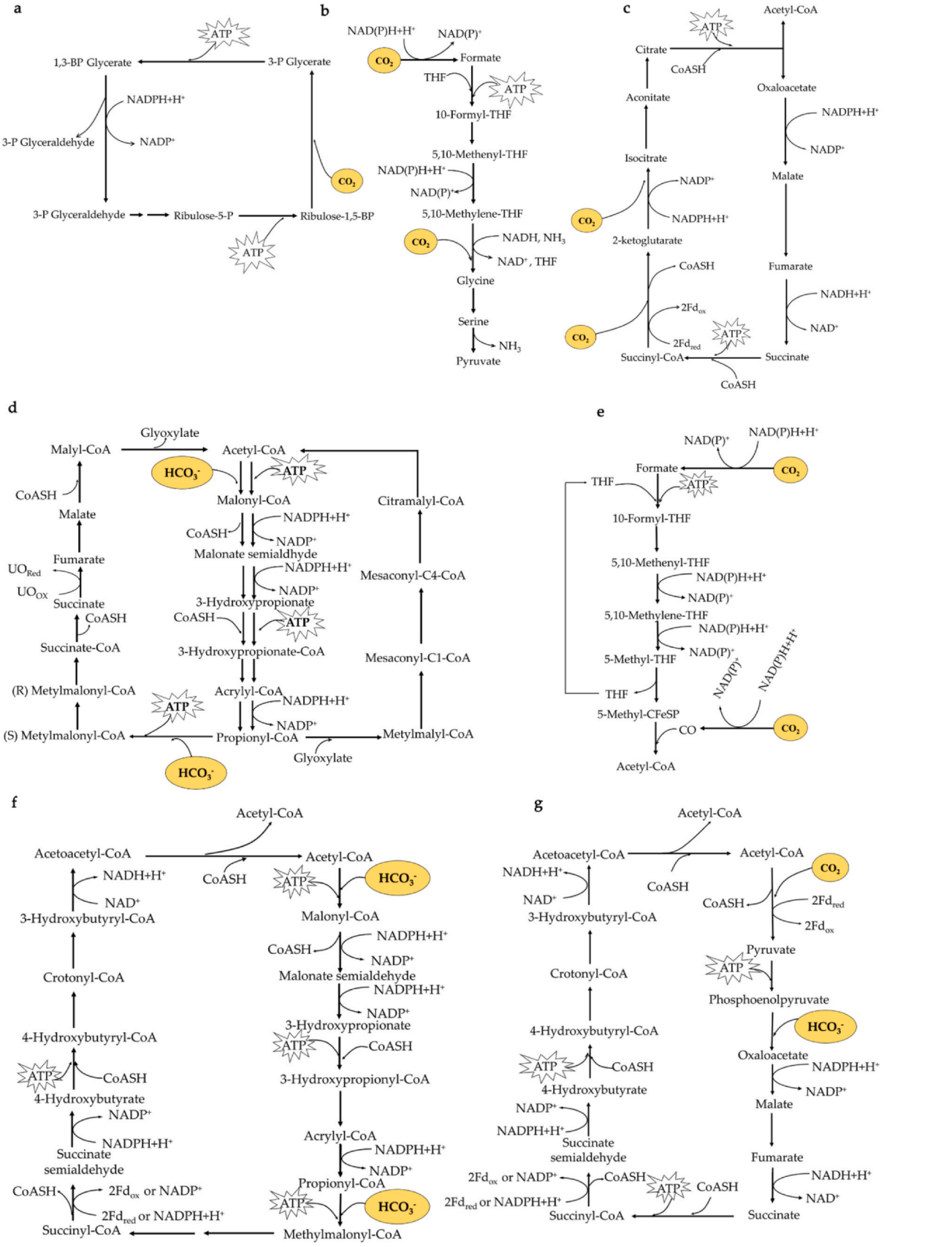

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Fig. 3. Natural carbon fixation pathway. a, The CBB cycle, which can direct reduce and fix CO_2. It is a universal carbon fixation pathway in nature. This cycle is closely related to the pentose phosphate pathway. b, The reductive glycine pathway, which could co-utilize CO_2 and formate to synthesize glycine. c, The reductive TCA cycle, which fixes two moles of CO_2 by reversing the oxidative TCA cycle. d, The 3-HPA cycle, which assimilates two moles of bicarbonate via acetyl-CoA/ propionyl-CoA carboxylase. e, The Wood-Ljungdahl pathway, which can direct reduce and fix CO_2 in acetogens. f, The HP/HB cycle, which assimilates two moles of bicarbonate via acetyl-CoA/propionyl-CoA carboxylase. g, The DC/HB cycle, which fixes one mole of CO_2 via pyruvate synthase and one mole of bicarbonate via PEP carboxylase. The seven CO_2 fixation pathways above mentioned are linked to the central carbon metabolic pathway through glyceraldehyde 3-phosphate, pyruvate and acetyl-CoA.

assimilate bicarbonates rather than CO_2 into the acetyl-CoA/ succinyl-CoA cycles. The 3HPA bicycle and the HP/HB cycle both have high energy costs. Their survival during evolution is possibly because they can tolerate oxygen and can assimilate bicarbonate [6], which is important as the intracellular concentration of bicarbonate is much higher than that of CO_2 [74]. It is also interesting that the first part of both pathways are quite alike (Fig. 3), even though they evolved independently in *Chloroflexaceae* and archaea, suggesting the importance of these biotin-dependent carboxylase reactions during chemoevolution [18]. Key enzymes in the 3-HPA bicycle are propionyl-CoA synthase and malonyl-CoA reductase [75,76]. Propionyl-CoA synthase is a fusion enzyme that can catalyze three steps in the 3-HPA bicycle from 3-hydroxypropionate to propionyl-CoA. Structural analysis of propionyl-CoA synthase has identified its multicatalytic reaction chamber and active sites, and increased its carboxylation yield for CO_2 fixation [77]. Similarly, malonyl-CoA reductase also catalyzes multi-reactions, and this feature has been used to construct efficient cell factory for 3-hydroxypropionic acid [78]. The key enzyme in the HP/HB cycle is 4-hydroxybutytyl-CoA dehydratase [67], and few study characterizing or engineering this enzyme of the HP/HB cycle has been reported. Reactions in the HP/HB cycle have been expressed in the hyperthermophilic *Pyrococcus furiosus* to incorporate CO_2 for the production of 3-hydroxypropionic acid [79]. This study also showed a significant advancement in development of the complete HP/HB cycle that can reduce CO_2 to acetyl-CoA. So far, heterologous expression of neither the 3-HPA bicycle nor the HP/HB cycle yielded autotrophic growth.

In addition to the mentioned natrual pathways, there are many synthetic pathways that play a significant role in the process of CO_2 fixation. Some synthetic metabolic pathways are shown in Fig. 4. Using a hybrid chemical-biological pathway, researchers achieved a groundbreaking feat by synthesizing starch from CO_2 and hydrogen in a cell-free system [80]. Using a "building block" approach, they constructed an artificial starch anabolic pathway (ASAP) consisting of 11 core reactions. By calculating pathways, assembling modules, and optimizing three bottleneck-related enzymes through protein engineering, they achieved the conversion of 22 nanomolar CO_2 to starch within 1 min under the catalysis of a total catalyst per milligram, which is about 8.5 times higher than the starch synthesis rate in corn. In addition, many semiconductor materials with excellent biocompatibility have been developed to provide strong reducing power to drive carbon sequestration pathways of microorganisms [41]. For example, non-metal-nitrogen-doped carbon nanosheets can be used as catalysts for hydrogen evolution reaction, promoting *R. eutropha* to convert CO_2 into poly-β-hydroxybutyrate, as shown in Fig. 4b [81].

Recently, another synthetic pathway called the crotonyl-CoA/ ethylmalonyl-CoA/ hydroxybutyryl-CoA (CETCH) cycle has been reported for CO_2 fixation, and exhibited high CO_2 fixation efficiency and low energy cost *in vitro* [82]. However, since this pathway has not yet been demonstrated *in vivo*, we will not discuss this pathway in much detail. Among currently identified pathways, the 3-HPA bicycle can only be found in photosynthetic organisms, the CBB cycle could be found mostly in photosynthetic organisms but also in chemosynthetic organisms (e.g. the proteobacterium *R. eutropha* [46]), and the Wood-Ljungdahl pathway, the reductive TCA cycle, the DC/HB cycle and the HP/HB cycle could only be found in chemosynthetic organisms. It is remarkable that most chemoautotrophic pathways employ acetyl-CoA/succinyl-CoA cycles (i.e., the reductive TCA cycle, the DC/HB cycle, the HP/HB cycle, and the 3HPA cycle). One issue regarding succinyl-CoA involved pathways might be its heat-lability, which may result in a trapped accumulation of succinate and CoA [18]. One common issue with these carbon sequestration pathways is their relative length. In general, the more steps a biochemical pathway takes, the less efficient it tends to be overall. To address this problem, Xiao *et al.* designed a minimal artificial carbon sequestration cycle based on thermodynamic and kinetic calculations of biochemical reactions [83]. This cycle, called the POAP cycle, consists of only four steps catalyzed by pyruvate carboxylase, oxaloacetate acetyl hydrolase, acetic acid-CoA ligase, and pyruvate synthase. The two steps catalyzed by pyruvate synthase and pyruvate carboxylase in the four-step cycle are carbon fixation reactions. Each POAP cycle converts two molecules of CO_2 to one molecule of oxalic acid, consuming two molecules of ATP and one molecule of reducing power. In another work, Zeng *et al.* has developed a way, named ICE-CAP pathway, to co-utilize CO_2 and other high-energy C1 compounds (such as methanol or formaldehyde) without the need to add ATP and cofactors such as NAD(P)H [84]. This pathway achieves efficient synthesis of glycine, serine, and pyruvate using methanol and CO_2. Notably, the product concentration can reach levels of g/L. The work also shows the great potential of combining biocatalysis and chemical catalysis.

4. Commercial attempts of CO_2-based biorefineries

Several companies have joined forces to develop CO_2 utilization techniques. For example, Phycal and Algenol are marketing their algae based technologies to produce algae oil and ethanol from sunlight, CO_2 and saltwater [88]. LanzaTech has reported commercially production of ethanol from steel mill flue gas using *Clostridium autoethanogenum*, with full scale production plants under construction in China and Belgium [37]. Recently, LanzaTech described a breakthrough in developing a process that enables the conversion of CO_2 into acetone and isopropanol at an industrial pilot scale [89]. However, the development of CO_2 biorefineries has lagged far behind traditional bioprocesses, so that it is currently hard to justify what production volumes or productivities would be needed to be competitive with 1G and 2G biorefineries, and deep understanding and optimization of each step of this process is required, including the identification of efficient catalysts, judicious pathways, host species and operation models. There are several natural CO_2 fixation pathways identified that might be worth to investigate in the future.

A crucial factor limiting the advancement of the CO_2-based biorefinery is the high cost for research and development. The issue is often referred to as the “valley of death”. The carbon CO_2 is + 4 valence, requiring significant energy and reducing power to be converted into biomass or corresponding products [90]. This factor contributes to a significant portion of the entire research and development cost associated with carbon fixation. Therefore, reducing the energy expenditure required to fix CO_2 is the key to reduce research and development costs [6]. Currently, public supports on biorefineries largely depend on the petroleum price. This fluctuating funding environment is starving small bioscience companies, particularly through the toughest part of research development. Thus, current biotech companies still focus on productions of value-added products. For example, Sapphire Energy has been concentrating on omega-3 oils [6]. Overall, political-driven environmental incentives on CO_2 and waste treatment for production of energy-dense liquid fuels might overcome the economic challenges [91]. Government should initiate diversified funding opportunities and

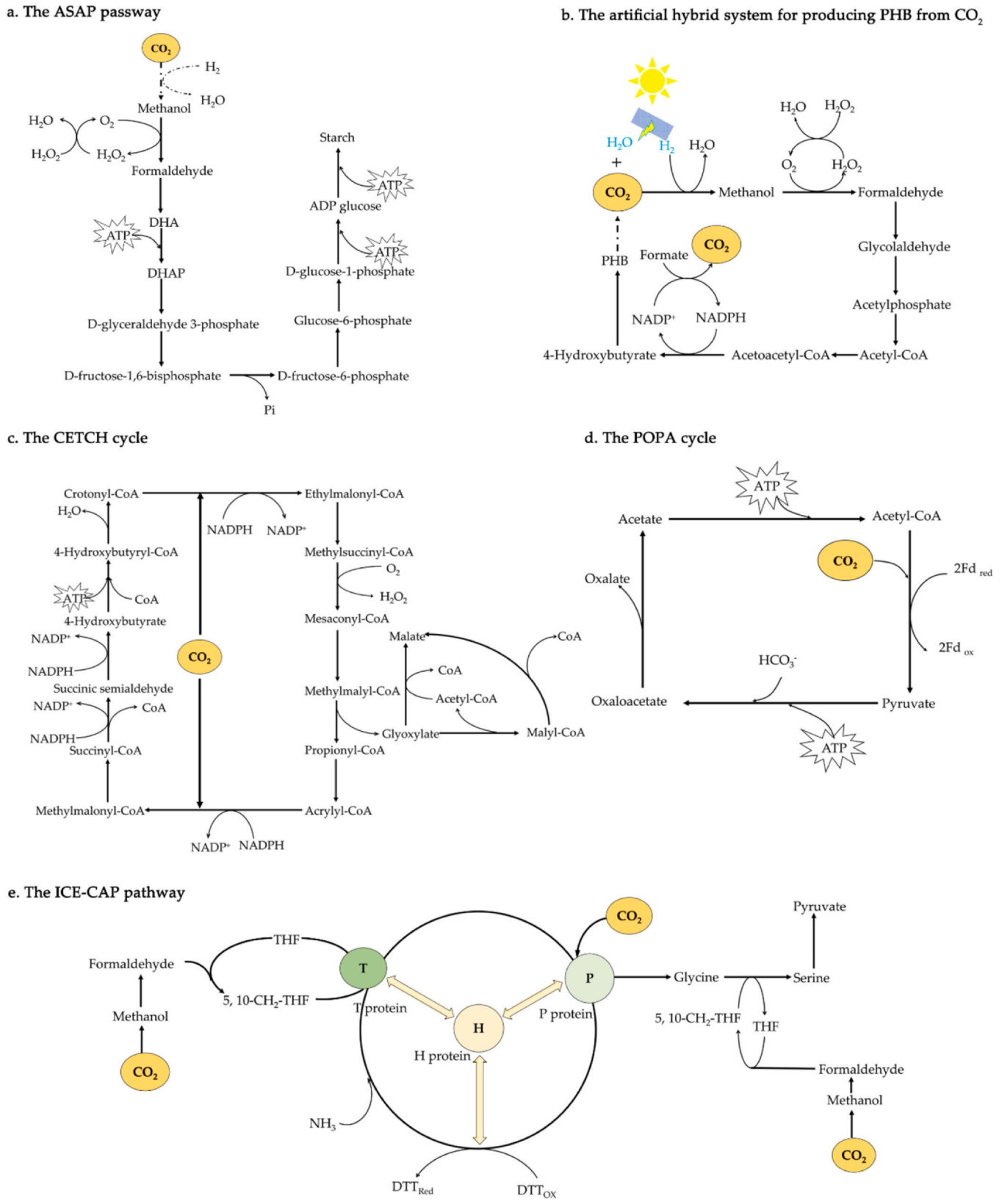

Fig. 4. Synthetic carbon fixation pathway. a, The ASAP passway, which convert CO_2 to starch. (The dotted line represents a multi-step reaction). b, The artificial hybrid system for producing PHB from CO_2. (The dotted line represents a multi-step reaction). c, The CETCH cycle. This cycle is a synthetic CO_2 fixation pathway verified *in vitro*. d, The POPA cycle, a minimal artificial carbon sequestration cycle. e, The ICE-CAP pathway, which operates without the need to add ATP and cofactors such as NAD(P)H.

provide revenue support to evaluate a variety of renewable energy sources for eventual benefits and risks without prejudices [92].

There are still many challenges in promoting the commercial application of CO_2-based biorefineries. The primary obstacles include the low carbon fixation rate and high energy requirements due to the low catalytic rate of carboxylase and the energy/carbon loss that occurs during the conversion of CO_2 into the cellular carbon metabolism center, which necessitates a significant amount of ATP [24]. Fortunately, advancements in biotechnology, especially synthetic biology, have led to the emergence of a number of technologies that can promote the development of CO_2-based biorefineris. For example, metabolic engineering strategies can be used to optimize the adaptation of carbon sequestration pathways to host's endogenous carbon and energy metabolism, including regulating the expression of enzymes related to carbon sequestration pathway [93] and enhancing the uptake and utilization of C1 substrate by chassis strains through laboratory adaptive evolution [20]. In addition, synthetic biology can be used to design and modify carbon sequester elements. Recently, the discovery of new enzymes such as PEP carboxylase [44] and crotonyl-CoA carboxylase [94], as well as investigations into the assembly and fine regulation mechanism of RuBisCO, can improve the catalytic performance of carbon capture enzymes [48]. Co-culture technology also serves as a powerful driving force for the development of the CO_2-based biorefinery. Studies have found that co-culture of *E. coli* and *Cupriavidus necator* can realize the conversion of CO_2 into sucrose, PHA and lipomacteric oligosaccharides [95]. Co-culture technology can not only effectively improve the efficiency of carbon fixation, but also enables the production of a more diversified products. Furthermore, the multi-disciplinary intersection has also injected more vitality into the development of the CO_2-based biorefinery, such as photobacterial coupling technology [96], electric bacterial coupling technology [97], AI-assisted protein design [98] and metal electrode materials capable of producing hydrogen negative ions [99].

5. Conclusions

For such a long time, the world is in a one-way fossil fuel diminution with little attention to waste reutilization and CO_2 fixation. Recent public concerns and research efforts have been devoted to shifting this situation to a more resource-conserving and environmentally friendly society. Specially, the recent CO_2-based bioeconomy proposed to circulate resources in a closed cycle to create a resource independent future [5]. However, the implementation of this technology still requires substantial research investment, because of the following limitations: (i) Many efficient CO_2 fixation hosts are either non-model organisms lacking tools for analysis of their physiology and for engineering their metabolism, or they grow slowly and require special cultivation conditions [35]. (ii) Most efficient CO_2 fixation hosts can only produce low amount of simple chemicals, such as formic acid, acetic acid, methanol. (iii) For all CO_2-based bioproduction processes, both the theoretical and practical yields are still far from industrial applications.

Microbial resources are abundant, and researchers could focus their efforts on discovering more carbon-sequestration microbes and developing additional carbon-using autotrophic strains to address the above problems. Exploring the potential of microorganisms in deep-sea hydrothermal vents for carbon fixation could be significant [100]. Genome sequencing represents a viable method for identifying new carbon-using strains, while CRISPR/Cas technology could greatly facilitate the genetic modification of model and non-model organism, expanding the range the organisms that can be genetically engineered with precision [101]. These technologies could be used to create more efficient strains for CO_2-based biorefineries.

Declaration of Competing Interest

Tianwei Tan is honorary editor-in-chief for Green Carbon and was not involved in the editorial review or the decision to publish this article. Jens Nielsen is an advisory board member for Green Carbon and was not involved in the editorial review or the decision to publish this article. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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