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Third-generation biorefineries as the means to produce fuels and chemicals from CO₂

3

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13

14 **Abstract**

15 Concerns regarding petroleum depletion and global climate change caused by greenhouse gas emissions have spurred
16 interest on renewable alternatives to fossil fuels. Third-generation (3G) biorefineries aim to utilize microbial cell
17 factories to convert renewable energies and atmospheric CO₂ into fuels and chemicals, and hence represent a route for
18 assessing fuels and chemicals in a carbon-neutral manner. However, to establish processes competitive with the
19 petroleum industry, it is important to clarify/evaluate/identify the most promising CO₂ fixation pathways, the most
20 appropriate CO₂ utilization models and the necessary productivity levels. Here, we discuss the latest advances in 3G
21 biorefineries. Following an overview of applications of CO₂ feedstocks, mainly from flue gas and waste gasification,
22 we review prominent opportunities and barriers in CO₂ fixation and energy capture. We then summarize reported CO₂-
23 based products and industries, and describe trends and key challenges for future advancement of 3G biorefineries.

24

There is an urgent need to switch from the traditional ‘take-make-dispose’ economy to a renewable economy with a reduced carbon footprint. The atmospheric CO₂ concentration remained stable at 200-280 ppm for 40,000 years¹, but in the last 50 years, the concentration has increased sharply to 414 ppm². This non-linear increase is still ongoing and it is likely that the CO₂ level will reach 500 ppm by 2045³, which may cause the Greenland and Antarctic ice sheets to melt, resulting in sea levels rising several meters⁴ and extinction of ~24% of plant and animal species⁵. Biotechnology offers environmentally friendly alternatives to produce fuels and chemicals in a carbon-neutral manner. For example, blending 10% bioethanol into gasoline could reduce emissions of CO₂, CO, NO_x and volatile organic compounds by 6-10%, 25-30%, 5% and 7%, respectively⁶. However, current bioproduction processes suffer from low energy conversion efficiencies and low productivities, thus a shift from sugar-based feedstocks (the first generation, 1G) and biomass (the second generation, 2G) currently in use to the use of atmospheric CO₂ (the third generation, 3G) is desirable.

3G biorefineries aim to use microbial cell factories to utilize atmospheric CO₂ and renewable energies, such as light, inorganic compounds from waste streams, electricity generated by sustainable sources including photovoltaic cells and wind power, for bioproduction. Compared with 1G and 2G biorefineries, 3G biorefineries substantially reduce the cost for feedstock processing and pose much lower security threats to food and water supplies⁷ and are thus starting to gain momentum. Great progress has been achieved to date; for example, eight natural and synthetic CO₂ fixation pathways have been validated, four energy capture techniques have been established, and several CO₂-based plants have been commercialized (Fig. 1). Key challenges of 3G biorefineries are the efficient fixation of atmospheric CO₂ and the efficient capture of the renewable energy for bioproduction. Autotrophs have evolved to support cell growth, but they may not produce the directed fuels or chemicals as efficiently under industrial conditions. To fulfil the goal of 3G biorefineries, autotrophs have been engineered to accommodate recombinant production, and CO₂ fixation pathways have been incorporated into heterotrophic microbial cell factories.

Here, we systematically analyse key components of 3G biorefineries. Briefly, we suggest that flue gas and gasification-derived gases are promising 3G feedstocks, although robust strains tolerant for high temperatures and toxic compounds are required. Moreover, we compile a comprehensive data set of current validated CO₂ fixation pathways, including oxygen sensitivity, ATP requirement, thermodynamics, enzyme kinetics, carbon species, and demonstrate that the Wood-Ljungdahl pathway and the 3-hydroxypropionate bicycle are the most suitable pathways for anaerobic and aerobic CO₂ fixation, respectively. We also analyse different energy capture techniques for 3G biorefineries, including

53 photoautotrophic synthesis, chemoautotrophic synthesis and autotrophic electrosynthesis, and suggest strains that are
54 most suitable for each technique. We then give an overview of current 3G biorefinery product, and end with a
55 discussion of future engineering directions (Fig. 2).

56 **3G Feedstocks**

57 The high feedstock cost, which normally accounts for more than 50% of the total cost of 1G and 2G biorefineries, is a
58 key reason why biorefineries often cannot compete economically with chemical processes^{8,9}. 3G biorefineries do offer
59 potential advantages because CO₂ is the most abundant carbon source on Earth, with 33 billion tonnes of anthropogenic
60 CO₂ emissions generated per year¹⁰. A challenge for CO₂ utilization is that most autotrophic cell factories grow slowly
61 using atmospheric CO₂ (0.04 vol.%), and although increasing CO₂ concentrations can improve cell growth,
62 concentrating CO₂ from ambient air is costly. On the other hand, current flue gas emissions and municipal solid waste
63 generation have reached 13.4 billion tons/year¹¹ and 2 billion tons/year¹², respectively. The flue gas and syngas
64 generated during waste gasification processes, typically contain 10-30 vol% CO₂^{13,14}, are suitable for culturing many
65 autotrophic microbial cell factories¹⁵ and will greatly decrease the cost of feedstock CO₂¹⁶. Several companies have
66 started to develop pilot-scale technologies based on flue gas and syngas fermentations; for example, East Bend station
67 utilizes high-sulphur-content flue gas from thermal power plants for the production of algal oil¹⁷. LanzaTech uses
68 proprietary *Clostridia* strains to produce ethanol, 2,3-butanediol and butanol from gases derived from steel mills, coal
69 production facilities and gasification processes^{14,18}. Electrochaea applies proprietary methanogenic archaea that are
70 robust against industrial flue gas contaminants to convert stranded electricity and off-gas from industrial processes
71 into pipeline-grade CH₄¹⁹. The utilization of flue gas and gasification-derived gases is limited by the fact that both types
72 of gas are at high temperatures (>100 °C) and to cool them to suitable temperatures for biocatalysts (~20-30 °C) is
73 costly. Moreover, concentrated carbon sources may contain toxic compounds, such as high concentrations of NO_x and
74 SO_x, and the growth of many organisms can even be inhibited by high concentrations of CO₂. Thus, the identification
75 of robust hosts and the development of detoxification pathways are necessary. Various species capable of utilizing
76 concentrated carbon sources have been identified. For example, *Methanothermobacter thermautotrophicus* is capable
77 of converting H₂/CO₂ (80/20) to methane at 65 °C²⁰; *Oscillatoria* sp. can utilize 100% CO₂²¹; *Chlorella fusca* LEB
78 111, isolated from coal power plants, can fix CO₂ at 0.36 g/L/day²²; and *Chlorella pyrenoidosa* can utilize high
79 concentrations of SO₃²⁻ (20 mmol/L) and NO₂⁻ (8 mmol/L)²³.

80 **3G carbon fixation pathways**

81 Nature has evolved diverse and sophisticated CO₂ fixation pathways over the last 4 billion years. To date, several CO₂
82 fixation pathways have been validated, and different theoretical pathways have been proposed. In the following, we
83 present these pathways and discuss their current limitations in terms of oxygen sensitivity, ATP use, thermodynamics,
84 enzyme kinetics, carbon species and concentrating mechanisms.

85 Validated CO₂ fixation pathways. Carbon fixation pathways that have been validated to date can be divided into six
86 groups according to their features such as topology, carbon fixation reactions and the carbon species being fixed (Fig.
87 3 and Table 1). The detailed chemistry of these pathways will not be elaborated here, as this review focuses on the
88 common and unique features of each pathway.

89 The Calvin-Benson-Bassham cycle (CBB cycle, also known as the Calvin cycle, or the reductive pentose phosphate
90 cycle)²⁴ is centred around carbohydrates and is closely correlated with the pentose phosphate pathway (Fig. 3a). The
91 key enzyme in the CBB cycle is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)²⁵, and the
92 overexpression of sedoheptulose 1,7-bisphosphatase also increases the photosynthetic rate and cell growth, suggesting
93 that this enzyme shares flux controls of the CBB cycle²⁶. The CBB cycle is used by most plants, algae, cyanobacteria
94 and proteobacteria²⁷. Recently, a complete CBB cycle was introduced into *Escherichia coli* and enabled the fully
95 autotrophy of cell growth solely from CO₂, using formate, which could also be generated from CO₂ electrochemically,
96 as the electron donor²⁸.

97 The Wood-Ljungdahl pathway (also known as the reductive acetyl-CoA pathway)²⁹ and the reductive glycine
98 pathway^{30, 31} are the only pathways that employ direct reduction of CO₂ (Fig. 3b and Table 1). The two pathways are
99 very similar in their topology; for example, CO₂ is first reduced and attached to a C1 carrier and then attached to
100 another CO₂ molecule to generate a C2 compound. Key enzymes in the Wood-Ljungdahl pathway are CO
101 dehydrogenase, formate dehydrogenase and formylmethanofuran dehydrogenase²⁷. The Wood-Ljungdahl pathway is
102 active in a variety of organisms, including euryarchaeota, proteobacteria, planctomycetes and spirochaetes²⁷. Recently,
103 Papoutsakis *et al.* expressed 11 core genes of the Wood-Ljungdahl pathway from *C. ljungdahlii* in *Clostridium*
104 *acetobutylicum* and reported that both CO₂ fixation branches are functional in *C. acetobutylicum*; however, the reaction
105 that connect the two branches needs further optimization³². In another study it was found that the rate limiting step in
106 the reductive pathway is catalysed by the reductive glycine cleavage complex³³. The reductive glycine pathway was
107 originally proposed as a viable synthetic pathway for CO₂ fixation³⁴. Recently, Figueroa *et al.* suggested that a natural

108 reductive glycine pathway might exist in the phosphate oxidizing bacterium *Candidatus Phosphitivorax anaerolimi*³⁰,
 109 yet a thorough biochemical analysis of this strain is still required to determine if the reductive glycine pathway exists
 110 naturally and can support autotrophic growth. The heterotrophic expression of the reductive glycine pathway for the
 111 production of cellular glycine and serine has been demonstrated in both *E. coli*^{33, 35-37} and *Saccharomyces cerevisiae*³⁸.
 112 However, the application of this pathway for autotrophic cell growth has not been reported.

113 The dicarboxylate/4-hydroxybutyrate (DC/HB) cycle³⁹, the 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle⁴⁰,
 114 ⁴¹, the 3-hydroxypropionate (3-HP) bicycle^{42, 43} and the reductive TCA cycle (also known as the Arnon-Buchanan
 115 Cycle)^{44, 45} have evolved around common intermediates. For example, these pathways all employ two conserved
 116 metabolites, succinyl-CoA and acetyl-CoA, and each cycle shares several reactions with another cycle in this group.
 117 These four pathways have been further divided into three groups based on the carbon species being fixed:

118 i) The DC/HB cycle fixes one mole of CO₂ via pyruvate synthase and one mole of bicarbonate via phosphoenolpyruvate
 119 (PEP) carboxylase (Fig. 3c). The key enzyme in the DC/HB cycle is 4-hydroxybutyryl-CoA dehydratase²⁵. This FAD-
 120 containing enzyme contains an oxygen-labile iron-sulphur centre, yet is adequately oxygen tolerant²⁵. To date, this
 121 cycle has been identified in anaerobes, including Thermoproteales⁴⁶ and Desulfurococcales³⁹, as well as in facultative
 122 aerobes, such as *Pyrolobus fumarii*, which grows under oxygen concentrations up to 0.3% and temperatures of
 123 ~106 °C⁴⁷. This pathway requires various iron-sulphur proteins and thioesters. To date, no heterologous expression of
 124 this cycle has been reported.

125 ii) The HP/HB cycle^{40, 41} and the 3-HP bicycle^{42, 43} assimilate two moles of bicarbonate via acetyl-CoA/propionyl-CoA
 126 carboxylase (Fig. 3d). Both cycles have very high energy requirements, and the reason these cycles have survived
 127 through evolution might be due to the fact that they can tolerate oxygen and assimilate bicarbonate rather than CO₂.
 128 The latter is advantageous, as the intracellular bicarbonate concentration can be much higher than the intracellular CO₂
 129 concentration. The key enzyme in the HP/HB cycle is also the 4-hydroxybutyryl-CoA dehydratase²⁵, and thus far, this
 130 cycle has only been found in aerobic crenarcheota²⁷. Recently, Keller *et al.* expressed 5 genes of the HP/HB cycle from
 131 *Metallosphaera sedula* in *Pyrococcus furiosus* and successfully produced 3-hydroxypropionate from H₂ and CO₂⁴⁸.
 132 On the other hand, the key enzymes in the 3-HP bicycle include malonyl-CoA reductase⁴⁹ and propionyl-CoA
 133 synthase⁵⁰. The 3-HP bicycle can be found in green nonsulphur bacteria²⁷. Recently, Way *et al.* divided the 3-HP
 134 bicycle into four subgroups and expressed each of them individually in *E. coli*, demonstrating that all subgroups can
 135 complement host mutations⁵¹. However, heterologous expression of all the 3-HP bicycle genes did not yet yield

136 autotrophic growth. Mitigating the deleterious effects on the cell growth of certain enzymes, such as methylmalonyl-
137 CoA lyase⁵¹, improving the reducing power supply possibly through electrosynthesis and optimizing the overall carbon
138 flux might be required to realize autotrophic growth on the 3-HP bicycle.

139 iii) The reductive TCA cycle fixes two moles of CO₂ by reversing the oxidative TCA cycle (Fig. 3e). The key enzymes
140 in the reductive TCA cycle include ATP-citrate lyase and 2-ketoglutarate synthase^{25, 52}. For a long time, it was believed
141 that citrate synthase catalyses the irreversible formation of citrate from acetyl-CoA and oxaloacetate. Therefore, for
142 autotrophic growth, citrate synthase has to be replaced by ATP-citrate lyase⁵³ or citryl-CoA synthetase together with
143 citryl-CoA lyase⁵⁴. Two recent studies have identified that, in both *Desulferella acetivorans*⁵⁵ and *Thermosulfidibacter*
144 *takaii*⁵⁶, natural citrate synthases can catalyse both the forward and reverse reactions. However, whether these citrate
145 synthases can support cell growth in recombinant hosts remains unclear. The reductive TCA cycle can be found in
146 proteobacteria, green sulphur bacteria, and aquificae bacteria²⁷. Liu *et al.* incorporated the reductive TCA cycle into
147 the periplasm of *E. coli*, and doubled malate production from glucose⁵⁷. To date, no heterologous expression of this
148 pathway for autotrophic growth has been reported.

149 The crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle represents the synthetic CO₂ fixation
150 pathway verified *in vitro* (Fig. 3f)^{58, 59}. This pathway reconstitutes a total of 17 enzymes originating from 9 organisms.
151 The CETCH cycle fixes CO₂ via crotonyl-CoA carboxylase/reductase, which is much faster than RuBisCO⁶⁰, and can
152 efficiently fix CO₂ using 40% less energy than the CBB cycle (Fig. 3a)⁶¹. Recently, Stoffel *et al.* further investigated
153 this crotonyl-CoA carboxylase/reductase, and reported that 4 amino acids are critical for the high activity and exquisite
154 selectivity⁶². Besides the CETCH cycle, Schwander *et al.* proposed more theoretical cycles of similar efficiency that
155 are all centered on enoyl-CoA carboxylation, and it will be exciting to see whether the rest of the proposed pathways
156 are functional, both *in vitro* and *in vivo*⁵⁸. Nonetheless, this study provides proof of concept for the feasibility of
157 synthetically designing and constructing synthetic carbon fixation pathways, which may fundamentally enable custom
158 design of 3G biorefineries in the future.

159 Theoretical CO₂ fixation pathways. In addition to elucidating existing CO₂ fixation pathways, efforts have also been
160 made to identify synthetic pathways. Genome analysis of a number of autotrophs has revealed that some species, such
161 as *Ferroplasma acidiphilum* and *Pyrobaculum arsenaticum*, do not possess genes from any of the known CO₂ fixation
162 pathways⁶³, indicating that there may be additional autotrophic pathways that have not yet been identified. The
163 identification of potential CO₂ fixation pathways requires the genome sequencing of more organisms, preferably those

164 from isolated ecological niches, to determine if they use known CO₂ fixation pathways, as well as detailed biochemical
165 analysis followed by the reconstruction and validation of the biological pathways and networks.

166 In addition to the validated pathways described in the previous section, a number of theoretical pathways have been
167 proposed for CO₂ fixation. For example, a variant of existing carbon fixation pathways combining reactions of the 3-
168 HP bicycle (Fig. 3d) and the DC/HB cycle (Fig. 3c) has been suggested to fix one mole of CO₂ via pyruvate synthase
169 and one mole of bicarbonate via PEP carboxylase to generate glyoxylate in only six steps (Fig. 4a)⁶⁴. Similar pathways
170 were once proposed to operate in *Chloroflexus aurantiacus*, but this idea was later abandoned. With the rapid
171 development of genomic and biochemical models, as well as synthetic biology tools, it might be worthwhile to evaluate
172 this pathway again. Moreover, Bar-Even *et al.* used a modeling approach to analyze 5000 metabolic enzymes, and
173 explored possible alternative pathways based on topology, ATP efficiency, kinetics and thermodynamic feasibility⁶⁵.
174 Based on this they proposed two malonyl-CoA-oxaloacetate-glyoxylate (MOG) pathways (Fig. 4b and Fig. 4c)
175 fixing two moles of bicarbonate via PEP carboxylase to generate glyoxylate. The MOG pathways borrow the
176 mechanism that naturally evolved in C₄ plants, in which carbon is first fixed by PEP carboxylase to generate
177 oxaloacetate, and to malate, then malate is decarboxylated to pyruvate to complete the futile cycle"; but in MOG
178 pathways, the released CO₂ is used by PEP carboxylase rather than by RuBisCO as it is in the C₄ cycle. It has been
179 suggested that the MOG pathways are better than the CBB cycle (Fig. 3a) in terms of pathway specificity, kinetics and
180 ATP efficiency⁶⁵.

181 In summary, all three theoretical pathways are based on the advantages of PEP carboxylase in terms of high specific
182 activity and superior affinity toward bicarbonate. Moreover, these pathways all generate glyoxylate rather than the
183 central metabolites commonly produced by natural carbon fixation pathways, such as acetyl-CoA and pyruvate. The
184 elucidation of the functionality of these pathways may provide valuable insights in fundamental research.

185 **Key factors in CO₂ fixation pathways.** To achieve high yields in the eight validated CO₂ fixation pathways,
186 understanding the mechanism of each pathway is crucial. To enable comparative analysis, we map the overall
187 stoichiometry of the conversion of CO₂ to acetyl-CoA in each pathway, and the results are presented in Table 1.

188 *Oxygen sensitivity.* One differentiating factor for CO₂ fixation pathways is the ability of the pathway to operate in the
189 presence of oxygen. The oxygen-sensitive enzymes in CO₂ fixation pathways include CO dehydrogenase/acetyl-CoA
190 synthases, pyruvate synthases, ferredoxin-dependent 2-ketoglutarate synthases and some metal-dependent formate
191 dehydrogenases^{25, 66}.

192 Generally, the Wood-Ljungdahl pathway (Fig. 3b) can only operate under strictly anaerobic conditions, possibly
 193 because it uses ferredoxins and an extremely oxygen-sensitive CO dehydrogenase/acetyl-CoA synthase; the DC/HB
 194 cycle (Fig. 3c) and the reductive TCA cycle (Fig. 3e) have oxygen-sensitive enzymes but can operate under both
 195 anaerobic and microaerobic conditions; in contrast, the CBB cycle (Fig. 3a), the reductive glycine pathway (Fig. 3b),
 196 the 3-HP bicycle (Fig. 3d), the HP/HB cycle (Fig. 3d) and the CETCH cycle (Fig. 3f) can all operate under fully aerobic
 197 conditions^{52, 67}. However, oxygen sensitivity varies substantially among pathways and organisms, enabling some
 198 oxygen-sensitive enzymes or pathways to function under aerobic conditions. For example, the CBB cycle in C3 plants
 199 is oxygen tolerant, however, RuBisCO may competitively oxygenates ribulose-1,5-bisphosphate and reduce
 200 photosynthetic efficiency by 20 to 50%⁶⁸. *Hydrogenobacter thermophilus* can utilize the reductive TCA cycle under
 201 aerobic conditions using hydrogen as the energy source⁶⁹. Similarly, aerobic *Sulfolobales* developed oxygen-
 202 insensitive DC/HB cycles using biotin-dependent acetyl-CoA/propionyl-CoA carboxylases rather than oxygen-
 203 sensitive pyruvate synthases^{25, 70}.

204 Aerobic autotrophic growth allows the biosynthesis of a wide range of products; however, special attention must be
 205 paid to improve the yield of this type of growth, as a large amount of hydrogen is required in O₂ respiration for ATP
 206 production. Anaerobic autotrophs, on the other hand, often suffer from low growth rates and low cell densities, or they
 207 are too ATP deprived to generate energy-intensive products⁷¹. Nevertheless, both aerobic⁷² and anaerobic pathways⁷³
 208 are reported to have industrially relevant titers and productivities.

209 *ATP requirements.* The required reducing equivalents, which are calculated based only on the number of electrons in
 210 the starting and ending compounds, are obviously the same in all CO₂ fixation pathways, whereas the ATP requirements
 211 are very different, varying from less than 1 to 9 moles of ATP equivalents per mole of acetyl-CoA formed. As shown
 212 in Table 1, the ATP efficiencies of the Wood-Ljungdahl pathway, the CETCH cycle, the reductive glycine pathway and
 213 the reductive TCA cycle are greater than those of the other pathways. These diverse ATP requirements can be partially
 214 explained by three factors: (i) Aerobic or anaerobic metabolism. Generally, pathways active under aerobic conditions
 215 consume more ATP than those active under anaerobic conditions, and the high amount of ATP can be provided by O₂
 216 respiration. (ii) The reducing equivalents and the electron donor⁷⁴. For example, ferredoxin ($E'^0 = -430$ mV) provides
 217 a higher energetic driving force than NAD(P)H ($E'^0 = -320$ mV); therefore, the replacement of NAD(P)H with two
 218 ferredoxins provides an additional energetic driving of ~20 kJ/mol⁷⁵. Similarly, since the heat of combustion of H₂S
 219 ($\Delta H_c = 519$ kJ/mol) is higher than that of elemental sulfur ($\Delta H_c = 293$ kJ/mol), this electron donor/acceptor pair provides

220 additional energy of 226 kJ/mol for carbon fixation compared with the electron donor/acceptor pair $\text{H}_2\text{O}/\text{O}_2$ ⁷⁶.

221 Moreover, protein synthesis is also an energy-intensive process; for example, protein biosynthesis by the ribosomes
 222 requires 4 moles of ATP equivalents per mole of peptide bond formed and the degradation of 1 mole of peptide bonds
 223 requires another 1 mole of ATP⁷⁷. Long pathways engaging large enzymes are therefore expensive for the cell to
 224 assemble, and this may also have to be considered when CO_2 fixation pathways are to be expressed in an organism.
 225 As shown in Table 1, the six circular carbon fixation pathways have different reaction numbers, ranging from 8 to 18.
 226 The noncyclic Wood-Ljungdahl pathway (Fig. 3b) and the reductive glycine pathway (Fig. 3b) are not discussed here,
 227 because recycling the intermediates in the pathway requires additional reactions. Here, we assume all the enzymes are
 228 efficiently expressed and are saturated with their substrates, and based on a protein synthesis perspective, we suggest
 229 that shorter carbon fixation pathways are preferred, especially during heterologous expression. Of course, in reality
 230 the ATP requirements to synthesize carbon fixation pathways also depend on the kinetics of the different enzymes in
 231 the pathway. Thus, in terms of costs what is important is the relative catalytic efficiency, i.e. the k_{cat} per unit mass. As
 232 the detailed kinetic information varies among different host strains, this aspect will not be discussed further in this
 233 review.

234 *Thermodynamics.* Thermodynamics determines the feasibility of a pathway. Thermodynamically challenging reactions
 235 ($\Delta_r G' > 10 \text{ kJ/mol}$) in CO_2 fixation pathways are catalysed by 3-phosphoglycerate kinase in the CBB cycle (Fig. 3a);
 236 formate dehydrogenase in the Wood-Ljungdahl pathway (Fig. 3b) and the reductive glycine pathway (Fig. 3b); CO
 237 dehydrogenase and formylmethanofuran dehydrogenase in the Wood-Ljungdahl pathway; pyruvate synthases and
 238 pyruvate:water dikinase in the DC/HB cycle (Fig. 3c); 3-hydroxybutyryl-CoA dehydrogenase in the DC/HB cycle and
 239 the HP/HB cycle (Fig. 3d); succinate dehydrogenase in the 3-HP bicycle (Fig. 3d); ATP-citrate lyase, 2-ketoglutarate
 240 synthase and isocitrate dehydrogenase in the reductive TCA cycle (Fig. 3e); and methylmalonyl-CoA mutase in the
 241 CETCH cycle (Fig. 3f). Many of these reactions are redox reactions, especially CO_2 fixation reactions. To overcome
 242 these thermodynamic barriers, cells use combinations of the following strategies. (i) They can maintain the highest
 243 possible ratio of the concentrations of substrates to products⁷⁸. For example, the intracellular metabolite concentrations
 244 in *E. coli* vary by six orders of magnitude ($0.1 \mu\text{M}$ - 100 mM)⁷⁹, whereas a 10-fold increase in a single reaction precursor
 245 will decrease $\Delta_r G'$ by 5.708 kJ/mol. However, reducing the concentration of the product of one reaction might
 246 concomitantly reduce the rate of the reactions that utilize these chemicals as substrates, resulting in trade-offs between
 247 thermodynamics and kinetics⁶⁴. (ii) Cells can provide a strong reducing environment. For example, the standard redox

potential of NAD(P) is -330 mV (pH=7, I=0.25). Since the intracellular ratio of [NADH]/[NAD] can be lower than 0.002, the ratio of [NADPH]/[NADP] can be higher than 50^{80, 81}, and the intracellular concentrations of cofactors can range between 1 μ M and 10 mM⁸², NAD(P) can actually support both the forward and reverse reactions with compound pairs between -500 mV to -130 mV. This means that by adjusting the intracellular ratio of [NAD(P)H]/[NAD(P)] and their intracellular concentrations, NAD(P) can support both the oxidation and reduction of reactions from carbonyl to hydroxycarbon and from carbonyl to amine ($E^{\prime m} \geq -225$ mV)⁸². However, NAD(P)(H) can not change the reaction directions for reactions out of this range, such as reactions from hydroxycarbons to hydrocarbons ($E^{\prime m} \geq -15$ mV).

Considering the six natural CO₂ fixation pathways, Morgan *et al.* calculated the total energy demand for biomass production based on the thermodynamics and stoichiometric flux balance for photons in the light-harvesting reactions, moles of hydrogen and sulfur for the sulfur reductase reaction or moles of hydrogen for the ferredoxin hydrogenase reactions, and the heat of combustion of hydrogen, elemental sulfur and hydrogen sulfide⁷⁶. The calculations suggest that when neglecting the hydrogen cost, the three chemoautotrophic pathways, namely the Wood-Ljungdahl pathway (836 kJ/ mole CO₂), the HP/HB cycle (834 kJ/ mole CO₂) and the DC/HB cycle (612 kJ/ mole CO₂), produce the same amount of biomass in a more energy-efficient manner than the three photoautotrophic pathways, namely the reductive TCA cycle (2401 kJ/ mole CO₂), the CBB cycle (2439 kJ/ mole CO₂) and the 3-HP bicycle (3152 kJ/ mole CO₂)⁷⁶. However, unlike light, molecular hydrogen is not free, and when considering the hydrogen cost, which to date in the best scenario is 20% during thermosolar hydrogen production⁸³, the energy demands for chemoautotrophic pathways have to be multiplied by 5, and thus exceed the energy demands of photoautotrophic pathways. This study provides a quantitative understanding of different CO₂ fixation pathways, and future engineering could consider incorporating kinetics, differences in growth rates and the maintenance energy into their models to simulate the actual operation of CO₂ fixation for cell growth and production.

Enzyme kinetics. The employment of CO₂ fixation pathways with kinetically efficient enzymes is highly preferred. In many cases, the CO₂ fixation rate is too low to establish a commercial process. For example, the CO₂ fixation rate in cyanobacteria is only 1-5 mg/L/h, whereas an industrial process requires rates on the order of 1-10 g/L/h⁸⁴. The identification of efficient CO₂ fixation pathways and enzymes, as well as the engineering of the identified enzymes using model-aided engineering and directed evolution⁸⁵, are therefore of substantial interest. The kinetically favourable CO₂ fixation enzymes reported to date include pyruvate carboxylase ($k_{cat}/K_M = 4.12 \pm 0.3 \times 10^6$ /M/s) from the 3-HP bicycle (Fig. 3d), the HP/HB cycle (Fig. 3d) and the reductive TCA cycle (Fig. 3e)^{86, 87}; acetyl-CoA

276 carboxylase/propionyl-CoA carboxylase ($k_{\text{cat}}/K_M = 2.48 \pm 0.96 \cdot 10^4 / \text{M/s}$) from the 3-HP bicycle^{87, 88}; PEP carboxylase
 277 ($k_{\text{cat}}/K_M = 1.04 \pm 0.33 \cdot 10^6 / \text{M/s}$) from the reductive TCA cycle^{87, 89}; and crotonyl-CoA carboxylase/reductase
 278 ($k_{\text{cat}}/K_M = 1.31 \pm 0.3 \cdot 10^6 / \text{M/s}$) from the CETCH cycle (Fig. 3f)^{87, 90}, details of the calculation can be found in
 279 Supplementary Table 1. Most of these enzymes fix bicarbonate rather than CO₂ partially because of CO₂ is with low
 280 intracellular concentration and hard to activate. Crotonyl-CoA carboxylase/reductase, on the other hand, is the only
 281 reported CO₂ fixation enzyme with both high k_{cat} ($\sim 50/\text{s}$) and k_{cat}/K_M ($\sim 1.1 \cdot 10^6 / \text{M/s}$) values, and thus it has attracted
 282 increasing attention for further characterization and engineering.

283 However, a vast number of CO₂ fixation enzymes are kinetically inefficient and difficult to engineer. For example,
 284 RuBisCO is notoriously inefficient ($k_{\text{cat}} \sim 1\text{--}10/\text{s}$ and $k_{\text{cat}}/K_M \sim 1.5 \cdot 10^5 / \text{M/s}$)⁹¹, and it catalyses side reactions with O₂
 285 that under atmospheric conditions generally add 40-50% extra ATP and NADPH to the cost of CO₂ fixation⁹².
 286 Considerable efforts have been devoted to optimizing RuBisCO; however, even with 25 X-ray structures of different
 287 RuBisCO isoforms and vast achievements in computational tools⁹³, limited progress has been reported. Understanding
 288 the catalytic domain, active sites, and direct and long-distance amino acid interactions, is required for improving the
 289 kinetics of a given enzyme.

290 Carbon species and concentrating mechanisms. The carbon species used in 3G biorefineries include CO₂ and
 291 bicarbonate. The concentration of dissolved CO₂ in equilibrium with air (pH 7.4, 20 °C) is only 0.012 mM²⁵. Because
 292 this concentration is highly dependent on temperature and salinity⁹⁴, and most organisms are sensitive to high
 293 temperatures and salinities, it is difficult to optimize this value *in vivo*. On the other hand, the concentration of
 294 bicarbonate in equilibrium with air (pH 7.4, 20 °C) is 0.26 mM²⁵. This value is primarily dependent on the dissolved
 295 CO₂ concentration and the pH ($\text{pK}_a [\text{HCO}_3^-/\text{CO}_2] = 6.3$)⁹⁵ and can be even higher at the pH of seawater (7.8 to 8.2)⁴⁰.
 296 Therefore, carbon fixation reactions that use bicarbonate may be more efficient than those using CO₂. Bicarbonate-
 297 utilizing enzymes include PEP carboxylase, acetyl-CoA/propionyl-CoA carboxylase and pyruvate carboxylase.

298 An increase in the substrate concentration can improve the thermodynamics and enzyme turnover frequencies, as well
 299 as disfavor side reactions involving the enzyme. The concentration of CO₂ can be optimized through energy-dependent
 300 CO₂ capture mechanisms, including the use of CO₂-capture peptides that can form low-density structures with
 301 nanochannels and selectively absorb CO₂⁹⁶ as well as the use of CO₂ concentrating mechanisms (CCMs), including
 302 transmembrane bicarbonate pumps and transporters⁹⁷, and carbon-fixing organelle-like microcompartments with high

303 contents of carbonic anhydrase and carboxylase, such as pyrenoids in chloroplasts⁹⁸ and carboxysomes in
304 cyanobacteria⁹⁹. It has been proposed that in acetogenic bacteria utilizing the Wood-Ljungdahl pathway (Fig. 3b), even
305 when the other factors are tuned to the largest extent physiologically feasible, it is still necessary to increase the cellular
306 CO₂ concentration to at least 130 mM¹⁰⁰.

307 **3G energy utilization**

308 The assimilation of carbon from CO₂ (oxidation state 4) into biomass (oxidation state approximately 0) requires a large
309 amount of energy, which can be acquired from light, chemicals or electricity harvesting. Currently, 3G biorefineries
310 lag behind 1G and 2G biorefineries in carbon utilization speed; however, the energy conversion in 3G biorefineries
311 has already surpassed those of 1G and 2G biorefineries. For example, the overall energy conversion efficiency of solar-
312 to-biomass-to-products in 1G and 2G biorefineries is estimated to be only ~0.2%¹⁰¹, whereas the solar-to-product
313 efficiency of photoautotrophs is reported to be 1-3%¹⁰¹, the chemical (such as H₂)-product efficiency of
314 chemoautotrophs is reported to be ~7%¹⁰¹ and the solar-electricity-product efficiency of autotrophic electrosynthesis
315 can reach up to 9-10%^{102, 103}.

Light: Photoautotrophic synthesis. Photoautotrophic synthesis utilizes the energy of photons to convert CO₂ into organic compounds (Fig. 5a). Photosynthetic organisms can be divided into oxygenic organisms, such as plants, algae and cyanobacteria, and anoxygenic organisms, such as green sulfur bacteria. Oxygenic photosynthetic organisms mainly utilize the CBB cycle (Fig. 3a)¹⁰⁴, whereas anoxygenic photosynthetic organisms utilize a variety of different pathways, such as the CBB cycle in *Rhodobacter*¹⁰⁵, the reductive TCA pathway in green sulfur bacteria²⁷, and the 3-HP bicycle in *Chloroflexi*¹⁰⁶. Different types of photosynthesis absorb different wavelengths of light and hence, absorb photons with a range of energies. Oxygenic photosynthesis requires the absorption of light with shorter wavelength (176 kJ/mole of photons), whereas anoxygenic photosynthesis involves the absorption of light with longer wavelength (162 kJ/mole of photons)⁷⁶. Different photosynthetic pathways require different numbers of photons; for example, the oxygenic CBB cycle in algae requires 17.7±5.4 photons per CO₂ assimilated¹⁰⁷, and the anoxygenic reductive TCA cycle in chlorobium requires only 10±2 photons per CO₂ assimilated¹⁰⁸. Recently, *E. coli* expressing the proteorhodopsin photosystem¹⁰⁹ and *S. cerevisiae* integrated with light-harvesting nanoparticles¹¹⁰ were shown to use photogenerated electrons for cell growth and production, paving the way for photoautotrophic synthesis in industrial workhorse organisms. However, these photosynthetic biohybrid systems are still in the early stage of development, and the remaining challenges include the selection of biocompatible light-harvesting devices and the seamless interlinking of biological and nonbiological components¹¹¹.

Photosynthesis is inhibited by intense light and is, on the other hand, self-shadowing¹¹². Thus, developing methods ensuring dense photoautotrophic cultures receive sufficient sunlight is difficult, as closed cultures are very costly and open-pond cultivations is susceptible to contamination. Different methods for increasing light capture efficiency in photoautotrophs, including extending the wavelength of capturable light^{113, 114} and engineering host photosynthetic mechanisms, have been tested^{115, 116}. For example, Overmann *et al.* reported that green sulfur bacteria from low-light environments (<4 μE/m²/s) can utilize photosystem I to directly reduce NADPH and ferredoxin rather than using reverse electron flow, which would consume more energy^{117, 118}. Moreover, Wang *et al.* reported a pilot-scale biofilm-attached cultivation system for *Arthrospira (Spirulina) platensis*¹¹⁹. Under greenhouse conditions, the biomass productivity and CO₂ usage efficiency ($\frac{CO_2 \text{ input} - CO_2 \text{ output}}{CO_2 \text{ input}}$) of this system reached 38.3 g/m²/d and 75.1%¹¹⁹; for comparison, open-pond cultivations typically show 8-20 g/m²/d biomass productivity¹²⁰ and ~50% CO₂ usage efficiency¹²¹.

Chemicals: chemoautotrophic synthesis. Chemoautotrophic synthesis obtains energy by oxidizing electron donors in

the environment, such as waste streams and mining residues (Fig. 5b). Chemoautotrophs have been identified in various ecological niches, and they can efficiently fix CO₂ using a wide range of CO₂ concentrations under diverse and even extreme environmental conditions. To date, the 3-HP bicycle (Fig. 3d) has only been found in photoautotrophs, whereas the CBB cycle (Fig. 3a), the Wood-Ljungdahl pathway (Fig. 3b), the DC/HB cycle (Fig. 3c), the HP/HB cycle (Fig. 3d), and the reductive TCA cycle (Fig. 3e) have all been found in chemoautotrophs¹²². Moreover, recombinant soluble [Ni-Fe]-hydrogenases from *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) can complement *E. coli* mutants lacking an endogenous hydrogenase biosynthesis pathway¹²³. This report paves the way for establishing chemoautotrophic growth in *E. coli* by, for example, utilizing the Wood-Ljungdahl pathway for CO₂ fixation, endogenous membrane-bound Ni-Fe hydrogenases for ATP production through nitrate-dependent hydrogen consumption¹²⁴ and heterologous soluble NAD-reducing hydrogenases for NAD(P)H production¹²⁵.

Electron donors for chemoautotrophic growth include ammonia, hydrogen, reduced carbon (CO and formate), sulphur (S and H₂S), phosphate and ferrous iron¹²⁶. Claassens *et al.* systematically evaluated different electron donors based on their physicochemical properties (Table 2) and suggested that H₂, CO and formate are more attractive than others for reducing cellular electron carriers because they can be produced electrochemically, with low reduction potentials (lower than -400 mV) and high enzymatic utilization activities (more than 10 µmol NAD(P)H/min/mg enzymatic system)¹²⁶.

Electricity: autotrophic electrosynthesis. Autotrophic electrosynthesis uses electricity, which can be generated from a wide range of renewable sources, including light, wind, tidal, hydro, and geothermal, to convert CO₂ to fuels and chemicals in microbial systems (Fig. 5c). Currently, the CBB cycle (Fig. 3a)¹²⁷ and the Wood-Ljungdahl pathway (Fig. 3b)¹²⁸ have been observed in autotrophic electrosynthesis.

Depending on the energy delivery strategies, autotrophic electrosynthesis systems can be divided into direct-charge-transferring systems, in which microbes directly consume electrons required to convert CO₂ into organic compounds, and energy-carrier-transferring systems, in which microbes can tolerate electricity and consume electrically generated energy carriers to fix CO₂¹²⁹. Exoelectrogenic species, such as *Cupriavidus*, *Clostridia* and *Moorella*, can be used in low-driving-voltage direct-charge-transferring systems and exhibit unique and efficient machineries that facilitate electron transfers between the cell membrane and conductive surfaces^{130, 131}. On the other hand, in energy-carrier-transferring systems, low-driving voltages can be used to produce energy carriers, such as formate, hydrogen, carbon monoxide, methanol, methane, ammonia, sulphur species and ferrous salts, to support cell growth^{126, 132}. As discussed

in the previous section, H₂, CO and formate are attractive energy carriers for autotrophic electrosynthesis under anaerobic conditions. Because the reduction potential of CO₂/CO reaches -520 mV, CO can directly reduce cellular ferredoxins ($E'^0 = -430$ mV) and support the reductive carboxylation of acetyl-CoA to pyruvate ($E'^0 = -500$ mV). However, since H₂ and CO are flammable gases, their use as electron carriers under aerobic autotrophic electrosynthesis may cause safety concerns. We thus suggest that formate may represent a more promising energy carrier under aerobic conditions since it has a high solubility and a high redox potential but does not require an additional electron acceptor nor does it create safety concerns related to volatility.

Several factors are critical for the practical implementation of autotrophic electrosynthesis. (i) The identification of an appropriate host strain. For example, the metabolic environment, the electron survival and transfer rate, and the standard redox potential all affect the optimal driving voltage. (ii) The solubility and mass transfer rate of gaseous energy carriers¹³³. For example, the use of a biocompatible perfluorocarbon nanoemulsion as the H₂ carrier was reported to increase acetate electrosynthesis by 190%, resulting in the highest reported productivity (1.1 mM/h)¹³⁴. (iii) The CO₂ concentration in the electrolyser. CO₂ has a very low solubility, especially in salt-based electrolytes. To address this problem, Hass *et al.* reported a gas diffusion cathode that allows direct interaction with gaseous CO₂, and they achieved close to 100% Faradaic efficiency using a *Clostridium* system for conversion CO₂ to butanol and hexanol¹³⁵. (iv) The compatibility between the electrode and the microbes¹³⁶. For example, during electrosynthesis under aerobic conditions reactive oxygen species are produced at the cathode, and toxic metals can be released¹³⁷. Cornejo *et al.* developed an ultrathin silica membrane that could chemically separate the abiotic and biotic components at the nanoscale while maintaining their electrochemical interactions¹³⁸.

3G-based production

A wide variety of 3G-based products have been reported. For example, many photoautotrophs, such as microalgae and cyanobacteria, can assimilate CO₂ from freshwater, sea water and wastewater for the production of a wide variety of fuels and chemicals, including butyrate¹³⁹, pharmaceuticals¹⁴⁰, aromatics¹⁴¹, lipids¹⁴² and hydrocarbon fuels¹⁴³. Within chemoautotrophs, *Clostridium* species are attractive platforms for producing a wide range of products, including butanol (*C. carboxidivorans* and *C. acetobutylicum*), 2-oxobutyrate (*Clostridium aceticum*) and 3-butanediol (*Clostridium autoethanogenum* and *C. ljungdahlii*)^{126, 144}. *C. necator* is also a very attractive platform, as it can produce polyhydroxy butyrate at a rate of up to 1.55 g/L/h in amounts of up to 70% of the dry weight¹⁴⁵. Recently, autotrophic electrosynthesis had started to gain momentum for production of fuels and chemicals, including ethanol (0.18 g/L/d)¹⁴⁶,

isopropanol (0.157 g/L/d)¹⁴⁷, butanol/ isobutanol (0.013 g/L/d)¹⁴⁸, acetate (18.72 g/L/d)¹⁴⁹, butyric acid (0.21 g/L/d)¹⁵⁰, caproic acid (0.95 g/L/d)¹⁵¹ and α -humulene C15 (0.036 g/L/d)¹⁵². Several companies have already established pilot or commercial plants based on 3G biorefinery processes (Table 3). For example, Fermentalg and Pond Technologies have used microalgae to autotrophically produce commercial dietary supplements and food ingredients, and LanzaTech and INEOS have used acetogens to commercially produce ethanol.

When evaluating commercial production, the key question is what productivity is required for 3G biorefineries to become competitive with production from fossil fuels. The answer to this question depends on the product of interest. The price of algae-based biofuels is currently estimated to be \$200/gallon, whereas petroleum diesel only costs \$2.6/gallon¹⁵³. A large component of the price of 3G biorefineries comes from CO₂ capture and transportation, biomass harvesting, as well as water and nutrient supplies¹³. It has been suggested that if the productivity of photoautotrophic algae biofuels from flue gas reaches 15 g/m²/d, the generated fuel could be economically competitive with ultralow-sulphur diesel¹⁵⁴. Similarly, regarding autotrophic electrosynthesis, it is estimated that the cost needs to be decreased by more than 80% to compete with current industries^{155, 156}. Overall, for 3G biorefineries to be competitive, the electricity costs should be decreased to below 4 cents/kWh, the energy conversion efficiency should be increased to 60%, and the specific fuel productivity should target 0.5 g/g dry weight/h^{157, 158}. Today, onshore wind power auctions in several countries have reached a cost of only 3 cents/kWh¹⁵⁹, and a recent development in biobased technology for hydrogen-to-electricity (H2e) conversion, called BioGenerator claimed to have reduced the cost to slightly above 2 cents/kWh¹⁶⁰, laying a foundation for the commercialization of autotrophic electrosynthesis.

Outlook

3G biorefineries offer the opportunity to alleviate ecological and societal problems by circulating resources and CO₂ in a closed loop¹⁰⁴. Climate changes have increased the awareness of the need for alternative technologies for the generation of fuels and chemicals, and 3G biorefineries offer an opportunity to harvest and recycle CO₂. This development is supported by more than 53 carbon tax policies worldwide covering 19.8% of global greenhouse gas emissions¹⁶¹. However, considering the high costs and substantial time investment required for strain engineering and realization, further increases in social, political and economic incentives are still needed. Fluctuating funding environments often cause small companies to fail, particularly during research and development phases. Therefore, most current biotechnology companies concentrate on the production of high-value-added chemicals. For example, Amyris is marketing fatty acid-derived fine chemicals and cosmetics, and Sapphire Energy is producing omega-3 oils

such as DHA and EPA. To establish 3G biorefineries for fuels and bulk chemicals, governments must continue initiating diverse funding opportunities and providing revenue support for the evaluation of a variety of renewable energy sources¹⁶² and, more importantly, establishing or increasing carbon taxes to \$10-1000/ton^{163, 164}, as this will drive the development of alternative technologies. Moreover, precise and robust models including environmental impact models including life cycle assessment analysis of the overall impact of energy sources on ecosystems should be developed for all renewable sources¹⁶⁵.

It is difficult to judge which CO₂ fixation pathway is most efficient for cell growth and production because several of these pathways require special metal chaperones, suitable redox environments, and membrane systems for ATP coupling⁵². Moreover, the functionality of a pathway also depends on the host (the enzyme kinetics, the standard redox potential, and the expected intermediate concentrations), the cultivation conditions (oxygen level, use of electricity, pH level, and iron concentration), and the product (energy deprived or condensed). Generally, a heavy reliance on ATP consumption, the employment of many kinetically unfavorable enzymes, and strict thermodynamic limits can all lead to reduced cell growth and production³⁴. It has been suggested that among chemoautotrophic pathways, given the same input of H₂ or equivalent electrons the Wood-Ljungdahl pathway (Fig. 3b) could produce greater acetate and ethanol, followed by the reductive TCA cycle (Fig. 3e), the HP/HB cycle (Fig. 3d) and the CBB cycle (Fig. 3a)⁵². For more energy-demanding products such as butanol, given the same input of H₂ or equivalent electrons, the rTCA cycle could produce the most butanol, followed by the HP/HB cycle and the CBB cycle, and the Wood-Ljungdahl pathway hardly produces any butanol owing to its ATP limitations⁵². Taken together, we suggest that, of all the identified pathways, the Wood-Ljungdahl pathway (Fig. 3b) may be the most suitable pathway for anaerobic CO₂ fixation, especially during autotrophic electrosynthesis and the coassimilation of multiple C1 and C2 compounds, while the 3-HP bicycle (Fig. 3d) might be the most suitable pathway for aerobic CO₂ fixation. The reductive glycine pathway (Fig. 3b) and the reductive TCA cycle (Fig. 3e) might also be attractive for aerobic CO₂ fixation if cell growth can be supported on CO₂ alone (under fully aerobic conditions). Moreover, the CETCH cycle (Fig. 3f), with its relatively low ATP requirements and oxygen tolerances, is also an attractive option, if it can be shown to be suitable for autotrophic growth *in vivo*. However, no single pathway is perfect for all applications, and choice of the pathway will always depend on the target product and the process to be established. Alternatively, rewiring endogenous metabolic processes to better accommodate carbon fixation pathways and testing and optimizing additional artificial pathways *in vivo* and *in vitro* are recommended.

456 It is also difficult to specify ideal production hosts, but the following characteristics should be taken into account: the
457 feedstock tolerance (flue gas or waste streams), the culture conditions (open pond or closed conditions; fresh water,
458 waste water or sea water; the nitrogen source; and the energy source), the target products (oxidized or reduced forms,
459 valuable products or bulk chemicals, and product tolerance), the energy capture efficiency, the carbon fixation rate,
460 the cell growth rate, the production capability (theoretical yield, practical yield and productivity), the robustness to
461 contamination and environmental challenges, the cellular metabolic processes, the feasibility of genetic manipulation
462 and the stability. Compared with well-characterized model organisms, the challenges in engineering autotrophic
463 organisms may include their relatively slow growth, the lack of efficient engineering tools, or the high complexity of
464 the cultivation strategies, whereas the challenges in the integration of autotrophic pathways into model heterotrophs
465 may include the incompatibility of autotrophic energy systems and poor enzyme expression⁹². Aerobic autotrophs
466 might be better suited than anaerobic autotrophs for the synthesis of ATP-demanding products, while anaerobic
467 autotrophs might be more suitable than aerobic autotrophs for autotrophic electrosynthesis. Moreover, scale up remains
468 challenging, as difficulties associated with the supply of sufficient light for photoautotrophs, potentially explosive gas
469 mixtures (O₂, H₂, CO, etc.) for aerobic chemoautotrophs, and electron transfer efficiency for autotrophic
470 electrosynthesis remain. Here, we suggest that attractive host organisms include photoautotrophs such as *S. obliquus*
471 (which has already been used in a commercial process⁷²), *C. pyrenoidosa* (which can remove 95.9% of CO₂, 100% of
472 SO₂ and 84.2% of NO from flue gas¹⁶⁶), and *Synechococcus elongatus* (which has rapid autotrophic growth comparable
473 to the growth of heterotrophic *S. cerevisiae*¹⁶⁷); aerobic chemoautotrophs such as *C. necator*, which can store carbon
474 in the form of polyhydroxy butyrate; anaerobic *Clostridia* such as *C. ljungdahlii*, *C. autoethanogenum* and *A. woodii*,
475 which can achieve high carbon recoveries¹⁶⁸; and model organisms such as *E. coli* and *S. cerevisiae*.

476 Another valuable route being tested is the interlinking of multiple carbon fixation modules and the eventual integration
477 of multiple technologies from material science, chemical processes, biological systems and process development to
478 achieve closed-loop CO₂ fixation and utilization (Fig. 5d). For example, Liu *et al.* developed a semi-integrated CO₂
479 biorefinery platform by interlinking Si nanowire arrays with *S. ovata* for the photoelectrochemical production of acetic
480 acid, which was then fed to *E. coli* for the production of n-butanol, polyhydroxy butyrate, and amorphadiene¹⁶⁹.
481 Recently, Mohan *et al.* suggested an integrated CO₂ biorefinery system, including microalgae cultivation, anaerobic
482 fermentation, photobacteria biorefinery and electrosynthesis, as shown in Fig. 5d¹⁰⁴. First, microalgae are used to
483 photosynthetically produce algae oil and biomass as well as a wide range of value-added products. Then, the deoiled
484 algal biomass is used as the carbon source for anaerobic fermentation to produce volatile fatty acids, biohydrogen and

CO₂¹⁷⁰. Next, the resulting volatile fatty acids and CO₂ are used to produce bioelectricity and bioplastics through a photobacteria biorefinery process¹⁷¹. Finally, the effluent from the whole process is used to fix CO₂ in electrosynthesis, thus closing the carbon cycle. This integrated CO₂ biorefinery system provides exciting opportunities for closed-loop carbon utilization, as it may overcome the disadvantages of individual systems; however, this system requires several different processes to operate in concert, which is difficult to achieve with the current levels of understanding. We therefore foresee that even though integrated systems provide exciting alternatives for 3G biorefineries, their implementation is likely to follow the implementation of the other systems discussed above.

In conclusion, we believe that, despite the technological challenges and market entry barriers, with recent technological advancements, 3G biorefineries may significantly contribute to the establishment of a more sustainable society. Future research directions should consider a 3G biorefinery as a sequence of individual operations, including feed stock supply and tolerance, carbon fixation and utilization techniques, energy harvesting techniques, and strain and process engineering techniques.

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Author contributions

Z.L., T.T. and J.N. drafted the outline. Z.L., K.W., Y.C., T.T. and J.N. wrote the manuscript. T.T. and J.N. supervised the research. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Figure legends

820 **Fig. 1. Milestones in 3G biorefineries.** Since the discovery of the CBB cycle in 1948, eight natural or synthetic CO₂
821 fixation pathways have been identified, and substantial progress has been made in CO₂ fixation and utilization. For
822 example, in 2006, the CO₂ utilization plant was established, and it used microalgae to fix flue gas for biodiesel
823 production. In 2012, in addition to photoautotrophic synthesis and chemoautotrophic synthesis, a third energy
824 utilization technique for CO₂ fixation, electrosynthesis using microbial cell factories, was reported. Here, we discuss
825 3G biorefineries, which aim to convert renewable energies and atmospheric CO₂ into fuels and chemical, and we
826 review prominent technological opportunities and barriers.

827 **Fig. 2. Key steps in 3G biorefineries.** Overall, carbon fixation and energy capture are the two critical techniques for
828 3G biorefineries. CO₂ from various sources can be captured and fixed through different mechanisms, using energy
829 from light, chemicals and electricity. To date, a wide variety of 3G-based products have been reported, with several
830 commercial plants already running. However, public awareness and political support, including increased research
831 funding and carbon taxes, will be important for the further development of 3G biorefineries.

832 **Fig. 3. Existing CO₂ fixation pathways.** The eight identified CO₂ fixation pathways can be divided into six groups
833 according to their common and unique features: **a**, The CBB cycle (in brown). This cycle is closely related to the
834 pentose phosphate pathway; **b**, The reductive glycine pathway (in red), the Wood-Ljungdahl pathway in acetogens (in
835 black) and methanogens (in purple). These pathways involve the direct reduction of CO₂ and fix CO₂ through the C1
836 carriers; **c**, The DC/HB cycle (in green). This cycle fixes one mole of CO₂ via pyruvate synthase and one mole of
837 bicarbonate via PEP carboxylase; **d**, The HP/HB cycle (in orange) and the 3-HP cycle (in light blue). These cycles
838 assimilate two moles of bicarbonate via acetyl-CoA/propionyl-CoA carboxylase; **e**, The reductive TCA cycle (in blue).
839 This cycle fixes two moles of CO₂ by reversing the oxidative TCA cycle; **f**, The CETCH cycle (in light green). This
840 cycle is a synthetic CO₂ fixation pathway verified *in vitro*. The C₁ feedstock is highlighted with an orange oval. The
841 changes in the Gibbs energy were calculated using eQuilibrator (<http://equilibrator.weizmann.ac.il>; at pH 7, ionic
842 strength of 0.1 M, and reactant concentrations of 1 mM) and are shown in blue.

843 **Fig. 4. Theoretical CO₂ fixation pathways proposed.** **a**, A variant of existing carbon fixation pathways. This
844 proposed pathway combines reactions of the DC/HB cycle and the 3-HP cycle, aiming to fix two moles of carbon in
845 only six reactions. **b** and **c**, Two malonyl-CoA-oxaloacetate-glyoxylate pathways. These pathways aim to fix two moles
846 of bicarbonate via PEP carboxylase to generate glyoxylate. The changes in the Gibbs energy were calculated using

847 eQuilibrator (<http://equilibrator.weizmann.ac.il>; at pH 7, ionic strength of 0.1 M, and reactant concentrations of 1 mM)
848 and are shown in blue.

849 **Fig. 5. Sketch of the different energy utilization systems for 3G biorefineries** **a**, Light-supplied systems. These
850 systems include organisms such as algae and cyanobacteria that directly absorb light and microbial cell factories
851 equipped with light-harvesting devices such as CdS and gold nanoclusters (AuNCs). **b**, Chemical-supplied systems.
852 These systems obtain energy by oxidizing electron donor such as metal ions and hydrogen in the environment. **c**,
853 Electricity-supplied systems. These systems include direct-charge-transferring systems, in which microbes allow the
854 direct conversion of electrons and CO₂ into organic compounds; and energy-carrier-transferring systems, in which
855 microbes can tolerate electricity and consume electrically generated energy carriers to fix CO₂. **d**, Integrated CO₂
856 biorefinery systems. These systems aim to integrate multiple technologies, such as microalgae cultivation, anaerobic
857 fermentation, photobacteria biorefinery and electrosynthesis, to achieve closed-loop CO₂ fixation and utilization.

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Tables

Table 1. Key factors in CO₂ fixation pathways. All calculations are based on converting CO₂ equivalents to acetyl-CoA. The reducing power of 2 molecules of reduced ferredoxin is taken as 1 molecule of NAD(P)H, or 1 molecule of ubiquinol. ^aThe mean and standard deviation (if applicable) of the k_{cat}/K_M values obtained from BRENDA (<https://www.brenda-enzymes.org/index.php>) are presented. ^bThe CBB cycle originally produces glyceraldehyde-3-phosphate. Here, we assume that 1 molecule of glyceraldehyde-3-phosphate produces 1 molecule of acetyl-CoA, 1 molecule of CO₂ and 2 molecules of NADH. ^cThe reductive glycine pathway and the 3-HP bicycle originally produce pyruvate. Here, we assume that 1 molecule of pyruvate produces 1 molecule of acetyl-CoA, 1 molecule of CO₂ and 1 molecule of NADH. ^dThe glyoxylate produced by the CETCH cycle is not adjusted to acetyl-CoA when calculating the energy and reducing equivalents.

Topology	Carbon species fixed	Pathways	Total enzyme number	Key enzyme	k_{cat}/K_M value ^a (M ⁻¹ s ⁻¹)	ATP equivalents	NAD(P)H equivalents	Energy source	Oxygen tolerance	Reference
PP pathway related	CO ₂	CBB cycle ^b	11	RuBisCO	1.7 *10 ⁵	9 ^a	4 ^a	Light	Yes	25, 172
CO ₂ reduction pathways	CO ₂	Reductive glycine pathway ^c	5	Reductive glycine cleavage complex	-	2 ^b	4 ^b	-	Yes	33
	CO ₂	Wood-Ljungdahl pathway	8	Formate dehydrogenase CO dehydrogenase Formylmethanofuran dehydrogenase	0.23*10 ³ 8.7 *10 ⁶ -	<1	4	Hydrogen	No	27, 173, 174
Around central metabolites	CO ₂ , bicarbonate	DC/HB cycle	14	4- hydroxy butyryl-CoA dehydratase	0.14 *10 ⁵	5	4	Hydrogen And sulphur	No	25, 175
	bicarbonate	3-HP bicycle ^c	18	Malonyl-CoA reductase Propionyl-CoA synthase	4.84±2.98 *10 ⁵ -	7 ^b	4 ^b	Light and sulphur	Yes	49, 50, 176
	bicarbonate	HP/HB cycle	15	4- hydroxy butyryl-CoA dehydratase	0.14 *10 ⁵	6	4	Hydrogen and oxygen	Yes	25, 175
	CO ₂	Reductive TCA cycle	8	2-ketoglutarate synthase ATP-citrate lyase	0.23±0.01 *10 ⁵ 2. 3 *10 ⁵	2	4	Light and sulphur	Yes	25, 177
in vitro pathway	CO ₂	CETCH cycle ^d	17	Crotonyl-CoA carboxylase/reductase	0.11 *10 ³	1	4	-	Yes	58, 178

869 **Table 2. Relevant properties of electron donors for chemoautotrophic synthesis.** This table is adapted from tables published in Current Opinion in Biotechnology¹²⁶.

Donor	Redox potential (E')(mV)	Specific activity (μmol NAD(P)H/min/mg)	Carbon fixation/ C1 assimilation pathway	Aerobic autotrophy	Solubility	Cellular import	Microbial toxicity	Toxicity to humans or ecosystems	Flammability
H ₂	-410	10-100	Wood-Ljungdahl pathway CBB cycle	No Yes	Low	Passive	Low	Low	High
CO	-520	1000-10000	Wood-Ljungdahl pathway CBB cycle	No Yes	Low	Passive	Medium to high	High	High
HCOOH	-420	10-100	Wood-Ljungdahl pathway CBB cycle	No Yes	High	Passive	Medium to high	Low	Low
CH ₃ OH	-160 (CH ₃ OH to CH ₂ O)	0.1-1	Wood-Ljungdahl pathway	No	High	Passive/ extracellular	Medium	Medium	Medium
CH ₄	80 (CH ₄ to CH ₃ OH)	-			Low	Passive	Low	Low	High
NH ₃	+350	-	Wood-Ljungdahl pathway CBB cycle HP/HB cycle	No Yes Yes	High	Passive/ extracellular	Medium to high (NO ₂ ⁻)	High (NO ₂ ⁻)	Low
Fe ²⁺	+770 (pH 2) -240 (pH 7)	-	CBB cycle HP/HB cycle	No Yes	Low to medium	Extracellular	Medium	Low	Low
S ₀	-210	-	CBB cycle HP/HB cycle	Yes Yes	Low	Extracellular	Low	Medium	Low
S ²⁻	-270 (S ²⁻ to S ₀)	-	CBB cycle HP/HB cycle	Yes Yes	Low (S ₀)	Passive/ extracellular	High	High	Medium
HPO ₃ ²⁻	-650	1-10	Wood-Ljungdahl pathway	No	High	Transport (ATP neutral)	Low	Low	Low
Cathodic electrons	-	-	Wood-Ljungdahl pathway	No	-	Extracellular	Low	Low	Low

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872 **Table 3. Current CO₂ assimilation companies and their products.** This table is updated from the table published in Bioresource Technology in 2016¹⁷⁹.

Strategy type	Company name	Final product	Application(s)	Process	Development stage	Web link
CO₂ capture	Great Point Energy	Pressurized CO ₂	Enhanced oil recovery	Catalytic hydro methanation	Pilot scale	https://www.greatpointenergy.com/
	DyeCoo Textile System	Pressurized CO ₂	Dyeing of textiles	Supercritical CO ₂	Commercial	http://www.dyecoo.com/
	PRAXAIR	Cryogenic agent	Cooling in food industry	High-pressure gas cylinders	Commercial	http://www.praxair.com/
	CO ₂ Solutions' Inc. technology	Pure CO ₂	Solvent-based CO ₂ capture	Carbonic anhydrase enzyme	Commercial	http://www.co2solutions.com/en
3G biorefinery	LanzaTech	Ethanol	Renewable energy	Acetogens gas fermentation	Commercial	http://www.lanzatech.com/
	INEOS	Ethanol	Renewable energy	Gas fermentation	Commercial	https://www.chemicals-technology.com
	Fitoplancton Marino	Proteins	Food industry	Microalgae	R & D	http://www.fitoplanctonmarino.com
	Fermentalg	Fatty acids and proteins	Food industry	Microalgae	Commercial	https://www.fermentalg.com
	Oakbio	n-butanol and bioplastics	Energy/ Packaging	Oakbio's proprietary microbes	Pilot scale	http://www.oakbio.com/
	Phycal	Oil biofuel	Energy	Algae	-	http://www.phycal.com/
	Pond Technologies	Biofuels	Renewable energy	Microalgae	Commercial	http://pondtechnologiesinc.com/
	Cellana	Biofuels	Energy	Algae	Commercial	http://cellana.com/
	Algenol	Ethanol	Renewable energy	Microalgae	Pilot scale	http://www.algenol.com/

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