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Construction of microbial platform chassis for CO_2 utilisation

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Abstract

To achieve a circular bioeconomy, carbon streams can be utilised through microbial conversion to produce value-added compounds. Although some microorganisms are naturally able to grow on these renewable carbon sources and generate desirable molecules, significant engineering is required to develop platform chassis exhibiting attractive performance parameters for industrial-scale processes. Here, we provide a brief overview of the core considerations in chassis engineering for autotrophic bioproduction, including carbon and energy supply, in addition to emerging standards for rewiring metabolic pathways to enhance growth and biosynthetic capabilities. We highlight examples of successful strategies, placing emphasis on recent advances in engineering autotrophic capabilities in both native autotrophs and heterotrophs.

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Platform chassis, Synthetic biology, Autotrophy, CO_2, Formate, Bioeconomy.

Introduction

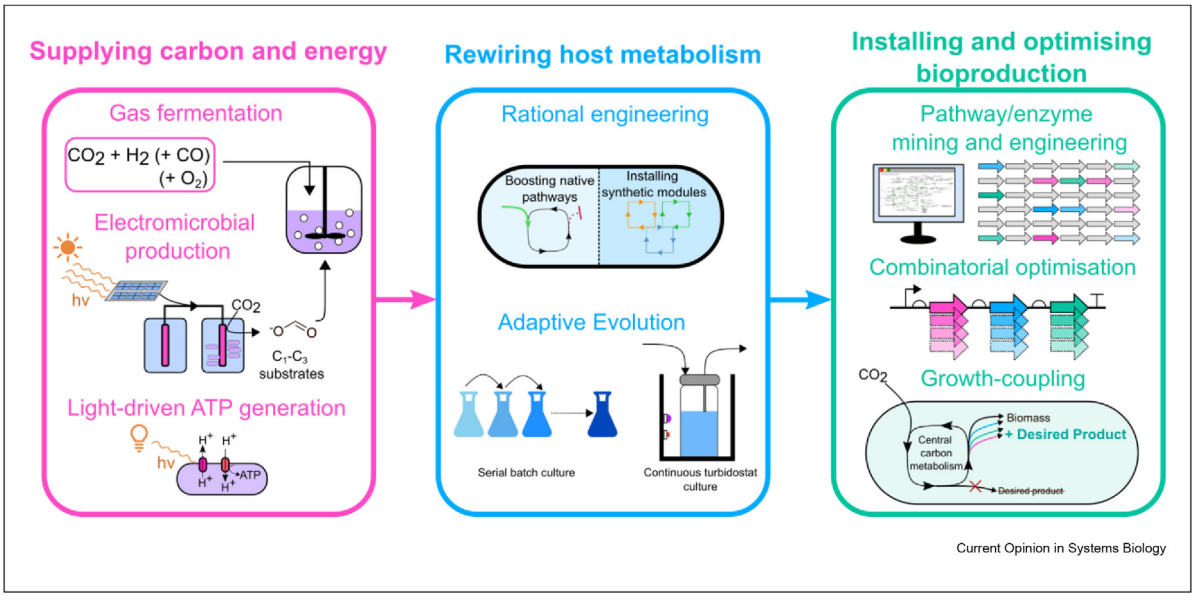
The accumulation of anthropogenic excess carbon in the environment is driving rapid change in planetary conditions, with adverse long-term consequences for both human society and biodiversity. Along with bioremediation approaches [1,2], the possibility of engineering biology to create microbial cell factories that can sequester and valorise waste carbon streams, particularly excess atmospheric carbon dioxide (CO_2), is steadily gaining prominence in both academic and industrial research arenas [3]. These microbial autotrophic hosts have the potential to deliver sustainable production of a variety of molecules, thus diminishing our reliance on petrochemical processing. Additionally, this approach could facilitate a shift away from the use of arable land to grow crops for industrial fermentation with heterotrophic microorganisms, further promoting environmental sustainability [4].

In recent years, a range of synthetic and systems biology approaches have been applied to engineer microbes that can utilise CO_2 and other one-carbon (C_1) substrates for growth and bioproduction. Studies have focused on addressing the long list of limitations associated with autotrophic hosts. These include inefficient carbon assimilation, low biomass yields, a narrow spectrum of target molecules suitable for bioproduction, and the scarce availability of efficient tools for genetic manipulation of emerging autotrophic model organisms.

General considerations for autotrophic chassis development

Though a variety of engineering strategies have been demonstrated, three key sequential considerations for developing any platform chassis for CO_2 valorisation can be broadly defined (Figure 1). Firstly, the optimal supply of both carbon and energy sources must be tackled, a consideration which is linked to host strain selection. Some industrially-relevant microorganisms, such as acetogens in the genus *Clostridium*, are amenable to direct cultivation on gaseous waste mixtures containing carbon monoxide (CO), CO_2, and hydrogen (H_2), a process widely known as syngas fermentation [5–7]. As an alternative to direct cultivation on gaseous CO or $CO_2 + H_2$, efficient abiotic (electrochemical) methods of CO_2 reduction into soluble C_1-C_3 compounds have been demonstrated [8,9]. These technologies allow for the decoupling of CO_2 reduction from its biological assimilation; a "reduction-first" approach which holds potential to boost the efficiency of C_1 bioprocesses [10], as well as to use a wider range of microbial hosts for CO_2 valorisation. The resulting reduced compounds, which are readily soluble and of low toxicity, can be used as effective electron mediators to support microbial growth and synthesis of value-added compounds in a hybrid process known as electromicrobial production. In particular, the use of electrochemically-derived formate

Figure 1



Strategies towards building microbial platform chassis for CO_2 valorisation. Arrows indicate the most common sequential flow between these three general considerations, though these optimisation strategies are often alternatively (and iteratively) connected. **Supplying carbon and energy.** Some chassis can be cultured directly on mixtures of gaseous substrates providing carbon (CO_2 and/or CO) and energy (H_2). In electromicrobial production, renewably-generated electricity can be used to power biohybrid autotrophic cultivation. Non-photosynthetic microbial hosts can also be endowed with light-harvesting capabilities to boost the supply of energy in autotrophic cultivation. **Rewiring host metabolism** can improve autotrophic growth kinetics. This can be attained via rational engineering or adaptive evolution, with both strategies often being synergistically combined. **Installing and optimising bioproduction.** Biosynthetic pathways for both native and heterologous products can be established and optimised using a wide range of tools. Successful strategies have leveraged bioinformatic searches to find promising production pathways and enzymes, as well as experimental optimisation through combinatorial assemblies and growth coupling.

has gained considerable interest in recent years, as it has numerous advantages over direct cultivation on CO_2 or syngas mixtures [11]. Formate is miscible, non-flammable, easily stored and transported, and simultaneously provides a source of carbon and energy, thereby bypassing the need to use H_2 as an energy carrier.

Secondly, the metabolism of the chassis is often rewired to enable autotrophic growth or increase its efficiency where native autotrophic capabilities are present. A wide array of natural and synthetic pathways for the assimilation of C_1 substrates have been explored and optimised towards achieving sustainable bioproduction goals, including the prevalent Calvin-Benson-Bassham (CBB) cycle, the Wood-Ljungdahl pathway (also known as the reductive acetyl-CoA pathway, rAcP), the serine cycle, and the synthetic-turned-natural reductive glycine (rGly) pathway. The natural and engineered architectures of these pathways, as well as their energy demands, have been extensively reviewed elsewhere [11–16].

Traditional metabolic engineering strategies have largely been driven by overexpression of key native or heterologous enzymes along carbon assimilation pathways. Recently, modular engineering approaches have emerged [17] and been applied extensively to improve autotrophy, most prominently to implement the rGly pathway in a range of hosts [18–22], as well as to install synthetic capabilities such as light-driven energy supply in non-photosynthetic hosts [23–26]. These diverse metabolic engineering strategies, which are often implemented at the genome scale, can be paired with adaptive laboratory evolution (ALE). As a systems-level tool, ALE enables *in vivo* exploration of strain optimisation landscapes beyond what is accessible by rational design alone. Recent reviews have detailed the methods available to implement systems-level metabolic engineering in combination with diverse evolution and selection strategies to develop efficient cell factories [27,28].

Lastly, bioproduction capabilities may be installed or optimised in the chosen chassis organism to convert assimilated CO_2 into value-added products. Whilst some autotrophic organisms can naturally synthesise useful compounds such as bioplastics, acetate or ethanol, efforts have been made to expand the range of industrially-relevant molecules that can be obtained through autotrophic fermentation. Despite the diversity in target compounds and biosynthetic pipelines, some

standardised engineering strategies can be identified for directing carbon into desired products. Notably, growth-coupled production has become an emerging standard [17,29,30]. Frequently supported by genome-scale metabolic models, which are increasingly available for non-model autotrophic organisms, growth-coupling principles intrinsically link the production of target molecules and biomass. This correlation ensures the evolutionary stability of engineered biosynthetic pathways and simplifies the Design-Build-Test-Learn (DBTL) cycle at the core of strain engineering [29].

Having discussed the fundamental considerations for autotrophic chassis engineering, we turn our attention to the most recent progress in the development of microbial platform chassis for autotrophic growth and bioproduction. Focusing primarily on advances reported in the past five years, we divide our analysis into two broad categories: the improvement of existing chemolithoautotrophic organisms, and the more challenging endeavour of installing autotrophic metabolism into traditionally heterotrophic hosts.

Engineering native autotrophic organisms towards improved growth and production

Naturally occurring chemolithoautotrophic organisms have evolved to fix CO_2, using inorganic compounds as electron donors. There are a limited number of biotechnologically relevant chemolithoautotrophic bacterial species. The most developed host organisms are acetogens such as *Clostridium ljungdahlii* and *Clostridium autoethanogenum*, alongside the bioplastic-producing bacterium *Cupriavidus necator* (formerly known as *Ralstonia eutropha*).

Acetogens assimilate CO_2 via the ATP-efficient Wood-Ljungdahl pathway, converting CO_2 to acetyl-CoA with H_2 as an electron donor. Due to their uniquely efficient mechanisms for energy conservation, acetogens have been described as operating at the thermodynamic limit of life [31]. A range of native products can be produced by autotrophic fermentation of acetogens on C_1 gas mixtures, such as ethanol, butyrate, 2-oxobutyrate, 2-3-butanediol, and acetate (reviewed comprehensively in ref. [32]). In addition, numerous studies have implemented production of non-native compounds in acetogenic hosts. The first successful report of genetic engineering in an acetogen to produce butanol was provided in 2010 [33]. Heterologous production of other industrially-relevant compounds, such as polyhydroxybutyrate (PHB) [34] and isobutanol [35], has since been attained. More recently, Liew and colleagues demonstrated carbon-negative production of acetone and isopropanol at industrial scale using an engineered strain of *C. autoethanogenum* [36]. Their novel approach relied on mining a diverse collection of biosynthetic enzymes, and assembling the enzyme sequences into a combinatorial library for pathway optimisation via high-throughput screening. The chassis microorganism was also optimised. Knock-out targets to improve production parameters were successfully identified using a combination of metabolic modelling and a streamlined cell-free method [37] for screening of native enzymes that may interfere with biosynthesis. The optimised biosynthetic enzymes and host strains were combined to deliver a scalable fermentation process with production rates of ~3 g/L/h at ~90 % selectivity. The report by Liew and colleagues provides a first-in-class demonstration of how synthetic biology tools can be applied to genome-scale optimisation of an autotrophic microbial cell factory to deliver truly sustainable and scalable biomanufacturing.

Acetogens have also been harnessed for electromicrobial production. In a demonstration of efficient "artificial photosynthesis", Haas and colleagues [38] used a CO_2 electrolyser module to continuously generate syngas. The electrolyser was directly coupled to a fermenter module containing a mixed population of the acetogens *C. autoethanogenum* and *C. kluyveri*. Combined, these species delivered the production of butanol and hexanol. The ground-breaking efficiencies reported in this study (100 % Faradaic efficiency and 78 % energy conversion efficiency of butanol and hexanol formation from syngas) provide evidence of the technical and economic potential of bio-hybrid systems. Moreover, the study demonstrates how the range of autotrophically synthesised products can be expanded by combining host organisms with distinct metabolic capabilities to form a bioproduction consortium.

The energetics of the Wood-Ljungdahl pathway limit the range of products that can be synthesised in acetogenic hosts. In contrast, aerobic chemoautotrophic hosts can mediate the synthesis of value-added chemicals requiring higher ATP investments. The facultative chemolithoautotroph *C. necator* has emerged as a promising platform chassis due to its versatile and malleable metabolism, as well as the increasing availability of tailored molecular tools to facilitate synthetic biology strategies [39]. Like most autotrophs, the bacterium assimilates CO_2 via a native CBB cycle. In addition to naturally-accumulated polyhydroxyalkanoates (PHAs), *C. necator* has been engineered to efficiently produce a wide array of molecules through autotrophic fermentation, such as sugars [40], alcohols [41,42], methyl ketones [43], terpenes [44], or alkanes [45]. Both wild-type and engineered *C. necator* strains have also been implemented within bio-hybrid systems for electromicrobial production. The first demonstration of this was provided by Li et al. in 2012 [41]. Here, *C. necator* was directly interfaced with a formate-producing electrochemical system to mediate the biotransformation of formate into isobutanol and 3-methyl-1-butanol. Interestingly, they observed that reactive oxygen and

nitrogen species generated during electrochemical CO_2 reduction inhibit bacterial growth. Though this limitation could be alleviated by shielding the anode with an inexpensive ceramic cup, the resulting total biofuel titer of 140 mg/l was 10-fold lower than the titer recorded using a formate-feeding fermentor.

Recent studies have sought to push the limits of electrochemical CO_2 reduction to formate and subsequent biotransformation by *C. necator*. Chen and colleagues demonstrated an innovative approach where an FDH enzyme in the cathodic chamber catalyses CO_2 reduction to formate, rather than the cathode itself [46]. To minimise the toxicity of reactive oxygen and nitrogen species as observed by Li et al., they also implemented physical separation of the cathodic and anodic chambers using a proton exchange membrane. A recent study by Lim et al. also provides support for securing live *C. necator* in a separate fermenter module to minimise adverse effects on bacterial growth [47].

Using a different iteration of electromicrobial synthesis, *C. necator* has been cultivated directly on CO_2 using H_2 generated by a water-splitting system [42,48]. These studies have demonstrated that, where the electrolyser module is powered by a solar-to-electricity device, solar-to-fuel efficiencies in the range of $\sim$10 % and $\sim$7−8% can be attained for biomass and value-added products, respectively. These efficiencies are well in excess of those attained by natural photosynthetic systems (1−3%).

Successful attempts to improve autotrophic metabolism in *C. necator* via the native CBB cycle have recently been demonstrated. This metabolic route for CO_2 fixation, which is most common amongst autotrophs, is relatively inefficient. The pathway operates with a high ATP demand, and its carboxylating enzyme (RuBisCO) exhibits a low catalytic rate, as well as a wasteful side-activity (oxygenation) which ought to be salvaged through the photorespiration pathway, further increasing the CBB cycle's expenditure of cellular resources [4,13−15,49]. Two independent reports have evidenced that the carboxylation bottleneck in the *C. necator* CBB cycle can be partially overcome by the heterologous overexpression of a cyanobacterial RuBisCO, boosting biomass and PHA yields [46,50]. Strains with improved autotrophic growth kinetics through the CBB cycle have also been obtained by ALE. Calvey et al. recently described a *C. necator* strain with a 24 % improvement in maximum growth rate on formate relative to the wildtype [51]. The strain was isolated through an innovative pipeline, combining evolution, -omics data, and rational genome engineering including genome reduction approaches. First, bacteria were cultured in a permissive formate concentration (50 mM) for a total of 400 generations. The best-performing individual isolates were identified and characterised by whole-genome sequencing, revealing a reduced set of unique and shared mutations which were hypothesised to contribute to the desirable formatotrophic growth kinetics. To discern the effects of individual mutations, these were recapitulated in a wildtype background. Several engineered strains were created, including genome-reduced variants where the organism's pHG1 megaplasmid was fully removed. Some of these strains exceeded the formatotrophic performance of even the best evolved isolates, providing further evidence for the synergistic combination of ALE and rational engineering approaches.

In an effort to overcome the inherent limitations of its native CBB cycle, *C. necator* has also been used as a sandbox for engineered autotrophy, most notably for the implementation of the synthetic reductive glycine (rGly) pathway. First proposed by Arren Bar-Even as the most efficient formate assimilation pathway for engineered autotrophy [15], the rGly pathway has since been identified in the naturally-occurring bacterium *Desulfovibrio desulfuricans* [52]. In a recent report, Claassens et al. successfully rewired the central carbon metabolism of *C. necator* to incorporate formate via the rGly pathway, thereby replacing the native CBB cycle entirely [18]. A modular engineering approach was implemented to accomplish this feat. First, a module allowing the conversion of formate to glycine via condensation with tetrahydrofolate (THF) was expressed. This required the expression of three heterologous enzymes to mediate flux from formate to 5,10-methylene-THF, as well as overexpression of native enzymes to mediate the conversion of 5,10-methylene-THF to glycine. The regulation and performance of this first metabolic module was optimised in a purpose-built *C. necator* strain exhibiting glycine auxotrophy. This approach represents an exemplary implementation of growth-coupled selection and modular optimisation of pathway designs. Next, glycine assimilation into biomass was investigated. Out of the two possible routes, proceeding either through glyoxylate or serine as intermediates, the latter is more efficient and therefore preferable. Flux was successfully forced through the serine route by overexpression of native enzymes. Following further strain optimisation through short-term ALE, the final "CRG4" strain obtained in the study exhibited a formatotrophic biomass yield similar to the wildtype. Since this groundbreaking report, work by Dronsella, Claassens and colleagues has further demonstrated that genomic integration of the modules encoding the rGly pathway pathway results in *C. necator* strains with formatotrophic biomass yields exceeding the wildtype (14 % yield increase) [19]. This work constitutes the first report of an engineered C_1-assimilation pathway surpassing the yield of an organism's native pathway, providing a

framework for the development of more proficient autotrophic host organisms through synthetic and systems biology.

Engineering synthetic autotrophy

The studies discussed thus far have unequivocally evidenced that engineered autotrophic platform chassis can mediate sustainable bioproduction. Yet these organisms are still relatively unknown to industry compared to their heterotrophic counterparts, such as *E. coli* or *S. cerevisiae*. In line with this, recent work in platform chassis development has focused on endowing well-established heterotrophic hosts with autotrophic capabilities, to expedite the use of (at least partially) autotrophic hosts for more sustainable industrial applications.

Heterologous expression of RuBisCO in *E. coli* has been explored as early as the 1980s [53]. The first example of carbon assimilation through an engineered CO_2 fixation module in *E. coli* was reported by the laboratory of Ron Milo in 2016. Here, hemi-autotrophic growth was successfully achieved by decoupling the metabolic modules needed for energy production and carbon assimilation [54]. Since then, work by the same group provided the first example of an *E. coli* strain capable of generating all biomass carbon from CO_2, using formate as an electron source [55]. Efficient assimilation of C_1 substrates in *E. coli* has also been reported by engineering a tetrahydrofolate cycle, similarly delivering strains that could grow on CO_2 and formate alone [56–58]. The rGly pathway has also been successfully implemented in *E. coli* to enable growth on formate and methanol [59,60,20]. In a paradigm-shifting study, Satanowski et al. demonstrated that autotrophic metabolism can be attained in *E. coli* not by engineering new pathways, but by rewiring its endogenous metabolism towards thermodynamically-feasible carbon assimilation pathways that do not require any heterologous enzymes [61]. Interestingly, work by the Savage group has proposed harnessing carbon-concentrating mechanisms to boost carbon assimilation in non-native autotrophic hosts. They demonstrated that heterologous expression of a 20-gene cluster from *Halothiobacillus neapolitanus*, encoding an α-carboxysome, was sufficient to allow CO_2-dependent growth of engineered *E. coli* in ambient air [62].

In addition to *E. coli*, two recent reports have demonstrated the possibility of engineering C_1 metabolism in the industrially-relevant bacterium *Pseudomonas putida* [21,22]. Both studies implement the tried-and-tested modular engineering approach to install the rGly pathway in this host, further highlighting the portability of this pathway and its potential to deliver autotrophy to novel platform chassis.

Eukaryotic microbes have also been subjects for engineered autotrophy. An early study showed that heterologous RuBisCO expression in *S. cerevisiae* could be harnessed to re-oxidise NADH using CO_2 as an electron acceptor, leading to an 8 % increase in ethanol production yields [63]. More ambitious metabolic rewiring towards autotrophy have also been explored in yeast. Notably, Gassler and colleagues successfully converted *Pichia pastoris* into an autotroph able to use CO_2 as sole carbon source [64]. Their novel approach harnessed the host's native peroxisomal xylulose monophosphate pathway, which enables methanol assimilation, as a background against which a heterologous CBB cycle could be installed. In an effort to expand C_1 substrate utilisation in industrial yeast strains, Zhan et al. implemented combinatorial pathway construction, modular engineering and ALE to successfully enable growth of *S. cerevisiae* on methanol as sole carbon source [65].

Powering autotrophy through engineered photosynthetic capabilities

A general challenge for engineering autotrophy, particularly in otherwise heterotrophic chassis, is to exploit efficient energy systems for regeneration of ATP and reducing equivalents. Recently, a series of studies have proposed addressing this limitation by endowing photosynthetic capabilities into non-photosynthetic hosts (autotrophs and heterotrophs). Two main strategies have been explored to enable bacteria to harness light energy effectively. The first strategy involves leveraging optogenetic tools, which harness light-sensitive proteins and can be engineered into non-photosynthetic bacterial hosts [23,24]. One prominent example of an optogenetic tool is the light-driven outward proton-pumping rhodopsin, which is the most abundant microbial rhodopsin [66]. These rhodopsins are widespread in marine bacteria and preferably absorb green light to generate the proton motive force for powering metabolic processes [66]. However, rhodopsin-based phototrophy cannot drive CO_2 fixation in the absence of an electron donor because the rhodopsin photoreaction does not involve any electron movement [67]. Previous studies mainly focused on rhodopsin facilitated ATP generation to enhance CO_2 fixation by using organic molecules as an electron donor [68,69].

A recent advancement integrates an electrochemical system with rhodopsin-expressing autotrophic *C. necator* to create a closed redox loop for photoelectrosynthesis [24]. Addressing the lack of electrons, this artificial photosynthesis system enables rhodopsin-dependent autotrophic growth using CO_2 as the sole carbon source [24]. In this system, the electrochemical module emulates photosystem II by enabling water splitting, while the incorporation of rhodopsin substitutes photosystem I

to drive the regeneration of ATP and NADH/NADPH. The second strategy is based on artificial photosensitisers by incorporating semiconductor-like nanoparticles into non-photosynthetic bacteria. These semiconducting nanomaterials, such as cadmium sulfide and indium phosphide, can be tailored to match the cellular microorganisms in terms of length scales and provide efficient light capture [25,26]. The nanoparticles are primarily anchored in the cell membrane, where they capture photons to generate reducing equivalent [70].

Autotrophic bacteria assembled with light-absorbing nanomaterials can harvest light energy to fix CO_2 into valuable chemicals. The distinction between nanoparticles and rhodopsin as light harvesters lies in the forms of biologically available energy they generate. Nanoparticles generate reducing power while rhodopsin powers ATP synthesis. Recent research combined self-assembled cadmium sulfide nanoparticles with rhodopsin in engineered *E. coli* with a synthetic CO_2 fixation pathway [71]. Under aerobic conditions, the increased ATP supply from rhodopsin reduces CO_2 emissions during glucose-based metabolism. Under anaerobic conditions, the reducing power generated by cadmium sulfide nanoparticles energises the CO_2 fixation pathway to maximise the conversion yield of carbon into value-added products (namely butyrate and L-malate). An outstanding limitation of this technology is that these nanoparticles can be cytotoxic, and are also quickly diluted during bacterial growth. Collectively, these studies show that the development of engineered photosynthetic capabilities holds great promise for powering autotrophic hosts to achieve efficient and sustainable bioproduction.

Conclusions and outlook

Autotrophic platform chassis, whether native or synthetic, hold the potential to catalyse a shift towards using C_1 substrates to produce valuable compounds. As highlighted in this review, a wide range of effective strategies have been implemented to construct microbial cell factories with attractive capabilities for sustainable bioproduction. These engineered strains have been made possible by significant advances in the fields of synthetic and systems biology, including tools such as combinatorial pathway construction and optimisation, cell-free prototyping systems, metabolic modelling, and evolutionary engineering.

However, it is important to acknowledge that some of the platform chassis developed so far, particularly fully synthetic autotrophs, exhibit poor performance, such as slow growth rates, long lag phases, and metabolite or energetic imbalances. Thus, further optimisation is required before these chassis can be efficiently applied to industrial processes. Additionally, some promising frameworks for CO_2 valorisation, such as electromicrobial production, face significant technical and economic challenges related to scale-up.

Nevertheless, considering the development of platform chassis with performance parameters that not only match but surpass native autotrophy, truly sustainable bioproduction from CO_2 and other C_1 substrates is closer than ever before. The coming years are expected to yield substantial advances in the field of CO_2 utilisation, with important implications for bioindustry and the broader shift to a circular carbon bioeconomy.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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