Synthetic biology strategies to address waste CO_2 loss during biofuel production

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ABSTRACT

Owing to differing degrees of reduction between substrates and products, biofuel production from carbohydrates inevitably releases a significant amount of CO_2, reducing both overall carbon utilization efficiency and the sustainability of biofuel production. To address this fundamental and persistent challenge, recent studies have explored diverse metabolic engineering and synthetic biology approaches to either i) limit CO_2 evolution by decreasing its generation or ii) recycle it through various biochemical mechanisms. Through these strategies, carbon that would have been wasted as CO_2 is minimized, allowing carbon conversion efficiency to be significantly enhanced and greenhouse gas emission effectively mitigated, thus leading to a more sustainable microbial process for biofuel production.

KEYWORDS:

Biofuel, CO_2, Synthetic Biology, Carbon Conservation, Sustainability

1. Introduction

Global climate change, driven by anthropogenic CO_2 emission, and concerns over unsustainable petroleum usage have stimulated efforts to develop renewable energy resources. Among the diverse renewable energy types, the predominant form (>60%) is biofuel [1], and microbial conversion of plant materials into fuel molecules represents a promising route to this end. To date, several inherent and persistent challenges still remain to limit the potential and cost competitiveness of such technologies. Prominent among these is carbon loss in the form of CO_2, ultimately caused by the differing degrees of reduction for plant-derived sugar substrates and fuel molecules, (e.g., ~49% of the mass of starting sugar is evolved as CO_2 during ethanol production). CO_2 evolved during microbial biofuel production leads to wasted substrate and reduced achievable production metrics, while further contributing to the greenhouse effect upon release. To minimize this carbon loss, diverse synthetic biology approaches have emerged to enable increased carbon conservation or active recycling of waste CO_2. Although still currently at the proof of concept level, these approaches have the ultimate potential to provide effective mechanisms for reducing carbon losses and minimizing greenhouse gas emissions, thus leading to more sustainable and economically-viable microbial biofuel production processes.

2. CO_2 loss during biofuel production

In 2020, the ethanol production capacity in the U.S. alone was about 17.4 billion gallons/year, produced by 201 biorefineries primarily from corn [2]. At its theoretical output (2.8 kg CO_2 per gallon of ethanol produced), this equates to approximately 49 million metric tons (MMT) of CO_2 per year. With very limited current merchant market opportunities for waste CO_2 derived from biofuel production [3, 4] the majority of waste CO_2 is emitted into the atmosphere.

Future biofuel production may move beyond corn ethanol and biodiesel derived from oil plants, to conversion of lignocellulosic biomass-derived sugars (predominantly D-glucose and D-xylose [5, 6]) – especially those from native perennial species grown on marginal lands [7] – into renewable fuels, which represents a sustainable route to meet ~20-30% of U.S. domestic energy needs. Furthermore, the U.S. Department of Energy predicts that ~1 billion dry tons biomass could be sustainably produced

domestically per year by 2030 without impacting food and feed markets [8]; a quantity representing the largest single feedstock for biofuel production. This increased biofuel production capacity in the future will face the same waste CO_2 challenge, which in turn will plague the overall sustainability of even advanced lignocellulosic biorefineries. As shown in ***Fig. 1***, fuel molecules are more reduced than sugars (higher degree of reduction, γ), and due to this redox constraint, production of biofuels is unavoidably accompanied with oxidized side-products, CO_2 mainly derived from pyruvate decarboxylation in this case, which occurs for both corn ethanol and all other emerging biofuels, including n-butanol, isobutanol, farnesene, fatty acids, and fatty acid-derived diesel molecules. Beyond fuel molecules, meanwhile, bioproduction of many other bioproducts (e.g., diols, long-chain hydrocarbons) that are also more reduced than carbohydrates thus faces the same challenge. As will be discussed, synthetic biology offers the potential to address the challenges of waste CO_2 byproduct, by minimizing production and facilitating its biological recycling (***Fig. 2***).

3. Synthetic biology strategies to address waste CO_2 loss

3.1 Carbon conservation through prevention of CO_2 generation

To address this fundamental challenge, recent studies have explored diverse synthetic biology strategies to rewire primary carbon metabolism to prevent CO_2 evolution, thus increasing carbon conservation (***Fig. 2A***) [9]. As a seminal example, non-oxidative glycolysis (NOG) was constructed in *Escherichia coli* by engineering glycolytic pathways to achieve non-oxidative conversion of one glucose molecule to three acetyl-CoA molecules instead of two [10]. Insufficient generation of reducing equivalents requires the provision of a reduced co-substrate (e.g., methanol) as additional electron donor [9]. Through follow-up efforts employing further strain engineering and adaptive laboratory evolution, nearly 100% carbon yield for glucose-to-acetate conversion was eventually achieved via NOG under aerobic conditions [11]. These engineered cells derive reducing equivalents and ATP from the TCA cycle and aerobic respiration, thus limiting the utility of NOG as an anaerobic production platform. In addition, considering the differing reduction degrees for sugars and biofuels (***Fig. 1***), the insufficient provision for reducing equivalent renders it incapable of supporting fermentative biofuel

pathways, which predominantly use reducing equivalents such as NADH for redox reactions. Recently, the critical carbon-conserving design of NOG (i.e., 'bifid shunt'; using bifunctional phosphoketolase to rearrange C5 metabolites for the production of C2 intermediates, such as acetyl-phosphate, without CO_2 loss), was combined with the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PPP) pathways to create the EP-Bifdo pathway in *E. coli* [12]. EP-Bifido strains showed improved product yield for polyhydroxybutyrate, mevalonate, and fatty acids, along with decreased CO_2 release [12]. , Sugar utilization and growth rates were significantly lower, which likely contributed to the observed reductions in the rates of productivity and CO_2 evolution, as well as achievable titers. Ongoing efforts to rewire primary metabolism for carbon conservation through the malyl-CoA-glycerate (MCG) pathway [13] and reversal of the glyoxylate shunt (rGS) [14] have also been reported. While increasing carbon conservation through these efforts, the significantly rewired primary metabolism introduces new production constraints and thus substantial follow-on efforts are required to adapt and implement these mechanisms into current biofuel production practices.

3.2 Metabolic engineering to actively recycle CO_2

Evolved CO_2 in biofuel production can also be fixed into value-added bioproducts (***Fig. 2B***). In nature, there are seven known CO_2 fixation pathways, among which the Calvin-Benson-Bassham (CBB) cycle is the most predominant mechanism commonly found in photosynthetic organisms [15-18]. Several studies have explored the use of native fast-growing photoautotrophs, such as algae and cyanobacteria, to fix CO_2 evolved from biofuel production and convert it to biofuel and other value-added products [19-21]. The rate-limiting CO_2 fixation step in CBB cycle employed in these processes is catalyzed by Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (RuBisCO). Even after billions of years of evolution, RuBisCO still has an extremely low catalytic efficiency [15, 22-25], which limits the application of CBB cycle for CO_2 fixation without further optimization. Recently, it was reported that an engineered *E. coli* was able to grow using only CO_2 and formate by introducing a functional CBB cycle as well as overexpressing formate dehydrogenase and phosphoribulokinase genes [26]. With further engineering, this mixotrophic platform could hold promise for carbon efficient biofuel production.

However, it is likely that the CO_2 fixation capacity in this strain will also be limited by the low catalytic efficiency of RuBisCO.

Besides implementing and modifying native CO_2 fixation pathways such as the CBB cycle [27, 28], reductive branch of TCA cycle [29], reductive glycine pathway [18, 30], 3-hydroxypropionate/4-hydroxybutyrate cycle [31], serine cycle [32], and tetrahydrofolate cycle [33] in heterotrophs, *de novo* design of synthetic CO_2 fixation mechanisms provides an alternative avenue with the potential to overcome existing constraints associated with native pathways. One noteworthy example is the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle [34, 35]; a synthetic CO_2 fixation pathway with theoretically lower thermodynamic barriers and ATP requirements relative to all native CO_2 fixation mechanisms [17]. As biological CO_2 fixation requires energy input, usually from redox reactions with diverse substrates including sugars, H_2, S, etc., or from light in photoautotrophs [17, 36], a chloroplast mimic was created to power the CETCH cycle (providing ATP and NADPH) by encapsulating photosynthetic membranes in cell-sized microfluidic droplets [34]. Despite the elegance of this approach, to fully explore the potential of this synthetic cycle an efficient strategy to provide energy to the CETCH cycle during large scale production is still needed.

3.3 Synthetic cocultures to increase overall carbon utilization efficiency

Compared to microbial monocultures, cocultures offer potential advantages such as strain-specific specialization, division of labor, and metabolic cooperation. Researchers have begun to explore the use of synthetic microbial cocultures for increasing carbon utilization efficiency during biofuel production by incorporating natural CO_2-fixing species (***Fig. 2C***). Strains with native CO_2 fixation pathways can be paired with biofuel producing microbes that release CO_2 to form cocultures that produce less emission and achieve higher carbon utilization efficiency. For example, *Clostridium acetobutylicum* (producing acetone, ethanol, and butanol while releasing CO_2) paired with *C. ljungdahlii* (using the Wood-Ljungdahl pathway to fix CO_2) together achieved increased overall substrate-carbon recovery [37]. Coculture stability was maintained by orthogonal catabolic functions without competition, with *C. acetobutylicum* using glucose and *C. ljungdahlii* using only H_2/CO_2. Similar

strategies have also been used to construct stable and catabolically orthogonal cocultures for efficient coutilization of lignocellulosic sugars [38, 39]. However, as the Wood-Ljungdahl pathway requires H_2 to fix CO_2, this demand for additional reducing equivalents potentially increases production costs and process complexity. Meanwhile, although often overlooked for their CO_2 fixation ability, anaplerotic reactions along with the reductive branch of TCA cycle are also suitable for this purpose in terms of overall low energy cost and suitable kinetic parameters for CO_2 fixation [15, 23, 24, 40]. However, such mechanisms have not been thoroughly investigated as a robust strategy for carbon conservation through CO_2 recycling in synthetic cocultures.

4. Future directions

Future research in following aspects may enhance current CO_2-conserving and CO_2-recycling strategies and likely push existing technologies closer to real applications:

First, in culture medium, HCO_3^-/CO_2 equilibrium is determined by pH, and at neutral pH (cytosolic and commonly used for bioproduction), CO_2 hydration to HCO_3^- is thermodynamically favored (-3.2 kJ/mol) with a HCO_3^-/CO_2 equilibrium ratio of 3.6 [41]; indicating that HCO_3^- is the predominant form of available inorganic carbon (C_i). Thus, poor rates for C_i cellular uptake presents as a potential bottleneck for CO_2 biological fixation. Synthetic biology approaches can be developed by engineering C_i uptake systems and carbonic anhydrase (catalyzing the interconversion between HCO_3^- and CO_2) to facilitate CO_2-fixing biochemical reactions inside the cell. Preliminary investigation using this approach led to enhanced carbon fixation in succinate-producing *E. coli* strains [42, 43]. Non-biological methods of improving CO_2 mass transfer, such as bubbleless gas-transfer membranes that increase delivery rates (and, subsequently, rates of photoautotrophic growth and CO_2 fixation) [44], might also prove useful.

Second, rates of biological CO_2 fixation are ultimately dictated by the properties of the primary C_i-fixing enzyme (i.e., RuBisCO in CBB cycle, acetyl-CoA synthase in Wood-Ljungdahl pathway, and other diverse carboxylases). Enzyme engineering through directed evolution and rational optimization offers the potential to increase the overall performance of both synthetic and native CO_2 fixation

pathways [45, 46]. In addition, plasmid-based overexpression systems were commonly used in the above-mentioned works, which is not ideal for bioproduction scenarios due to metabolic burdens associated with gene overexpression, costly chemical inducers, and strain stability [47]. Optimization and chromosomal integration of foreign genes involved in synthetic CO_2 fixation pathways in production hosts represent an important future direction. This is a common gap needed to be filled to allow laboratory concepts to advance closer towards real-world biofuel production scenarios.

Third, current CO_2-recycling strategies still rely upon the use of native mechanisms to provide the needed energy, including via either light or chemical redox reactions. The efficiency by which light energy is harvested using native photosystems has room to improve, as do chemical oxidations of substrates such as sugars, S and H_2. For example, more than 90% of the photon energy delivered to a photoautotrophic system is not harvested by current photosystems I and II [48]. It has also been proposed to use alternative energy sources to fix CO_2 such as electricity (engineering electroautotrophy) [36, 49], which is a new frontier for synthetic biology. Research to date in this direction is still at the initial phase [50]. As an emerging competing route to electroautotrophy, CO_2 can also be reduced and ultimately converted to useful products using electrochemical methods (CO_2 electroreduction) with the aides of nonbiological catalysts [51].

5. Conclusions

Biofuel production from carbohydrates is inevitably accompanied by significant carbon loss in the form of CO_2, yet the discussed synthetic biology approaches show promise in carbon conservation and CO_2 recycling. These approaches offer the important potential to increase sustainability and economic viability of biorefineries for biofuel production while effectively mitigating greenhouse gas emissions. Future efforts are expected to convert proof-of-concept demonstrations into real applications.

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Figure legends

Figure 1. CO_2 evolution during the production of different types of biofuel. Theoretical maximum mass yields are calculated and the values of the degree of reduction (γ) per carbon are shown for glucose and biofuel molecules.

Figure 2. Synthetic biology strategies to address waste CO_2 loss during biofuel production. A) The primary carbon metabolism can be rewired to prevent CO_2 evolution and thus increase carbon conservation. B) Native and synthetic CO_2 fixation pathways can be used to actively recycle waste CO_2 derived from biofuel production. C) CO_2-emitting biofuel producing microbes can be paired with CO_2-fixing microbes to form synthetic cocultures for improved overall carbon utilization efficiency through inter-strain CO_2-recycling.

Fig. 1

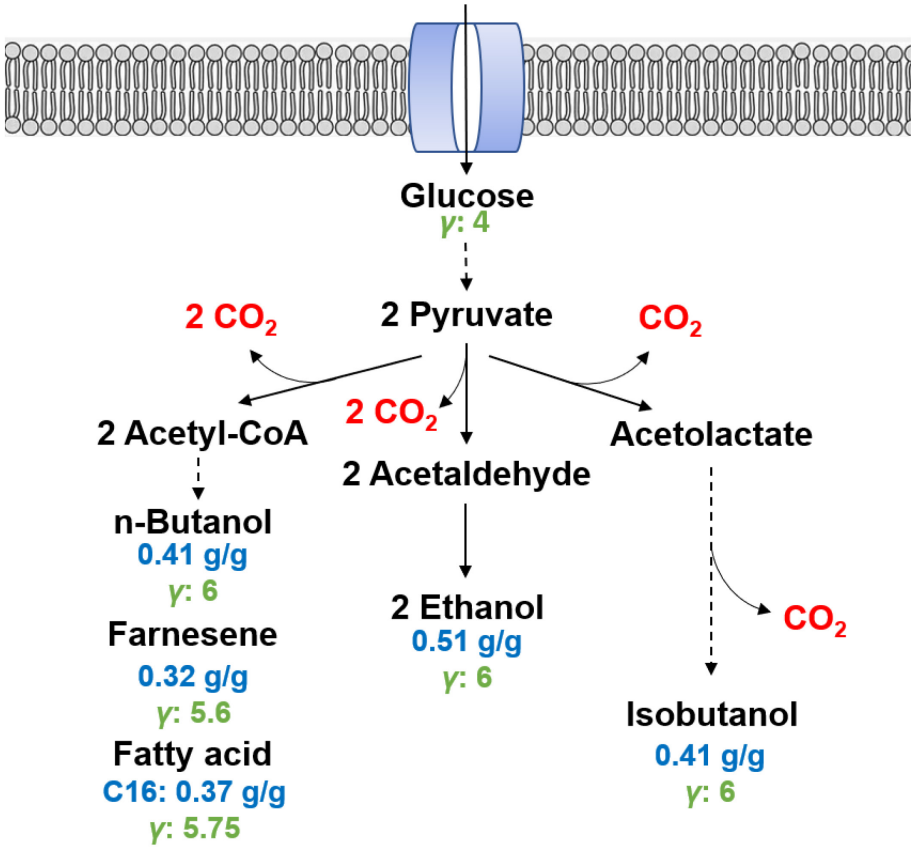
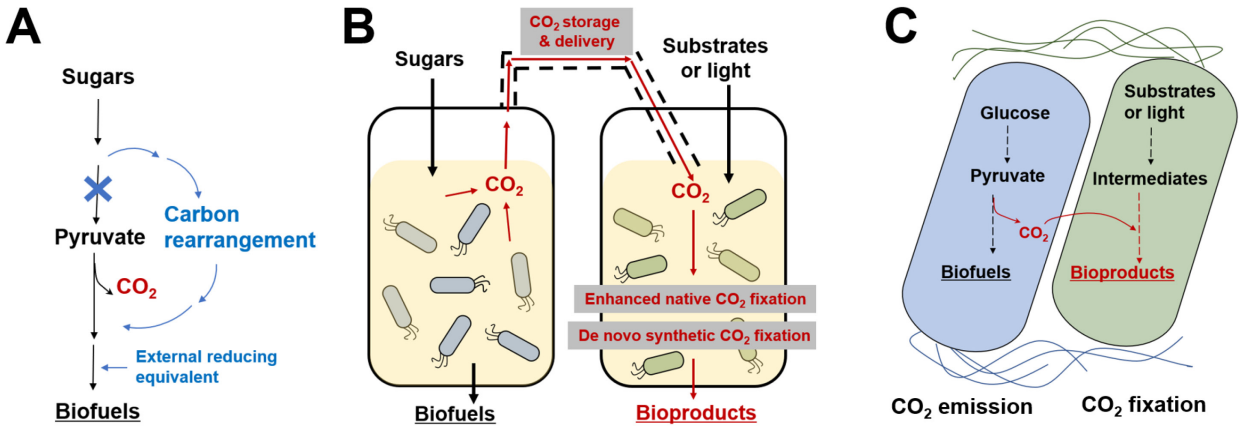

Glucose
γ: 4
2 CO2
2 Pyruvate
CO2
2 Acetyl-CoA
2 CO2
Acetolactate
2 Acetaldehyde
n-Butanol
0.41 g/g
γ: 6
Farnesene
0.32 g/g
γ: 5.6
Fatty acid
C16: 0.37 g/g
γ: 5.75
2 Ethanol
0.51 g/g
γ: 6
CO2
Isobutanol
0.41 g/g
γ: 6

Fig. 2


A
Sugars
Pyruvate
Carbon rearrangement
CO_2
External reducing equivalent
Biofuels
B
CO_2 storage & delivery
Sugars
Substrates or light
CO_2
CO_2
Enhanced native CO_2 fixation
De novo synthetic CO_2 fixation
Biofuels
Bioproducts
C
Glucose
Pyruvate
CO_2
Biofuels
Substrates or light
Intermediates
Bioproducts
CO_2 emission
CO_2 fixation

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