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Current Opinion in Biotechnology

Review

Optimizing *Cupriavidus necator* H16 as a host for aerobic C1 conversion

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Biological systems capable of converting CO_2 or CO_2-derived, single-carbon (C1) compounds can be used to reduce or reverse carbon emissions while establishing a circular bioeconomy to provide sustainable sources of the fuels, foods, and materials humanity relies on. A robust bioeconomy will rely upon a variety of microorganisms capable of assimilating C1 compounds and converting them to valuable products at industrial scale. While anaerobic microbes are ideal hosts for production of short-chain acids and alcohols, microbes capable of aerobic respiration are well suited for biosynthesis of higher molecular weight products. One such organism is the gram-negative soil bacterium *Cupriavidus necator*, which has been utilized in commercial production of biopolymers for decades. More recently, its capability of robust, aerobic growth on CO_2 has inspired research efforts that have advanced it toward becoming one of the leading bacterial hosts for C1-based biomanufacturing. This review highlights those efforts in the context of the characteristics that have historically made *C. necator* an excellent host for industrial bioconversion processes: its metabolic versatility, ability to grow rapidly to high cell densities, and genetic amenability.

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Current Opinion in Biotechnology 2025, **93**:103306

This review comes from a themed issue on **Gas Fermentation**

Edited by **Diana Machado de Sousa, Michael Koepke, Esteban Marcellin** and **Lars Keld Nielsen**

Available online xxxx

https://doi.org/10.1016/j.copbio.2025.103306

Introduction

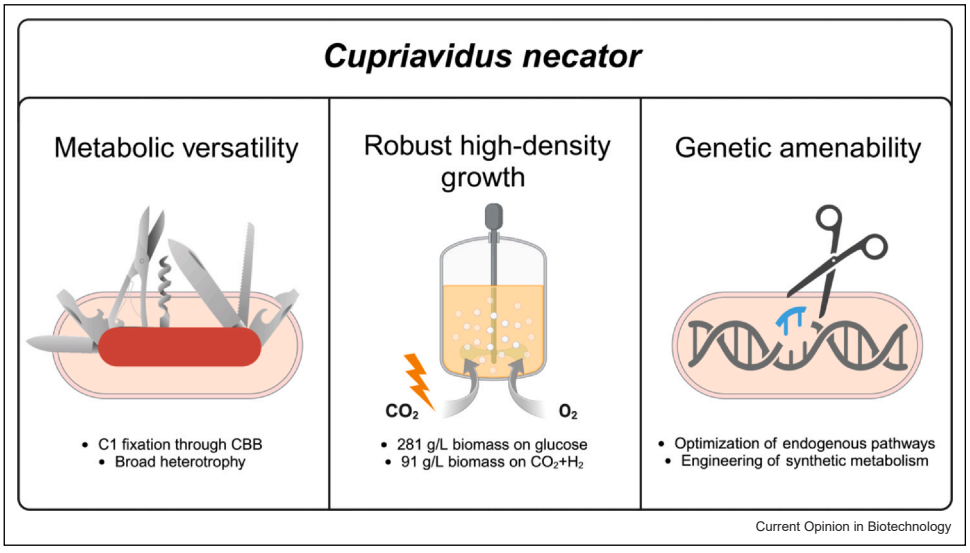
In the early 20th century, bacteria capable of growing aerobically on CO_2 using H_2 as an energy source began being identified, characterized, and assigned to the genus *Hydrogenomonas*. These included the Betaproteobacteria *Hydrogenomonas eutropha* H16 (also known as strain 337, DSM 428, ATCC 17699, and NCIB 10442), which was isolated from a soil sample in Göttingen, Germany, and described in 1962 [1]. Through a series of taxonomic reclassifications, this species has been known since as *Alcaligenes eutrophus*, *Ralstonia eutropha*, *Wautersia eutropha*, and, most recently, *Cupriavidus necator*, [2,3] named for its tolerance to copper and status as a predator of other bacteria; *Cupriavidus* is Latin for 'lover of copper' while *necator* means 'slayer' [4]. Upon isolation, it was also observed that under chemolithotrophic or organotrophic conditions, *C. necator* can accumulate large amounts of polyhydroxyalkanoates (PHAs), specifically polyhydroxybutyrate (PHB) [5]. PHAs are biodegradable polymers that are naturally produced by a variety of bacteria for carbon storage [6]. In the 1980s, Imperial Chemical Industries cultivated *C. necator* in bioreactors of up to 200,000 l in a 300-metric-ton-per-year commercial process, producing PHAs primarily from carbohydrates [7,8]. In the decades since, *C. necator* has been widely studied as a model for biosynthesis of PHAs and continues to be used for their commercial production [9]. More recently, concerns about CO_2 emissions have led to renewed academic and industrial interest in this organism's ability to assimilate and convert CO_2 and CO_2-derived C1 compounds. Here, we review the characteristics of *C. necator* H16 that make it a promising host for biomanufacturing: its metabolic versatility, robust, high cell density growth, and amenability to genetic manipulation (Figure 1) focusing on recent advances to hone these innate capabilities toward the valorization of C1 compounds.

Metabolic versatility

C. necator H16 is capable of growth on a wide range of substrates, including carbohydrates, organic acids, glycerol, aromatic molecules, and CO_2 [10,11], lending it utility for a variety of bioconversion processes. This metabolic versatility is supported by a large 7.41 Mbp genome [12,13] spanning two chromosomes, the first of which is thought to be ancestral and contain most essential genes [14], and a 0.45 Mbp megaplasmid, pHG1 [15].

C. necator H16 fixes CO_2 through a complete Calvin–Benson–Bassham (CBB) cycle using H_2 as a source of energy via the activities of a soluble (SH) and a membrane-bound (MBH) hydrogenase (Figure 2) [16]. Expression of these hydrogenases relies on the action of a regulatory hydrogenase and several maturation factors, all of which are encoded on the pHG1

Figure 1

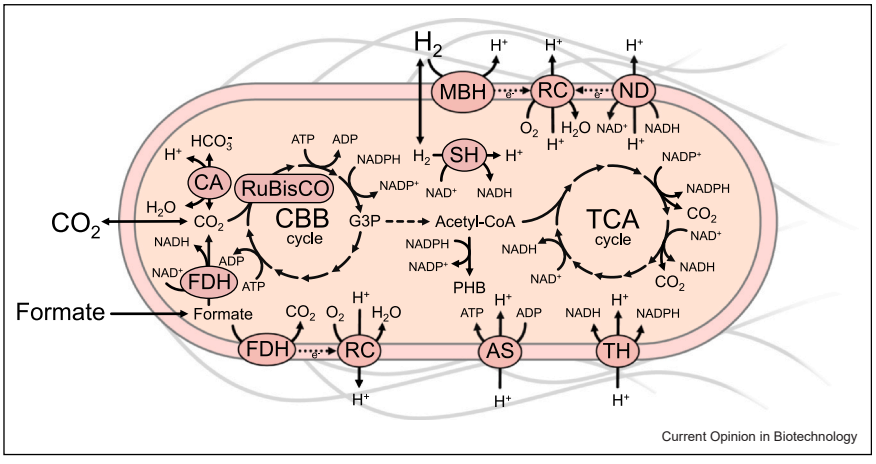


Characteristics that make *C. necator* a promising host for biomanufacturing. Created in BioRender. Donati, S. (2024) https://BioRender.com/m49a085.

megaplasmid [16]. Genes encoding the CBB cycle, including the key CO_2-fixation enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), are present in two copies, one on chromosome 2 and one on pHG1 adjacent to the hydrogenase genes. Interestingly, these genes are expressed [17] and active [18] even on a heterotrophic substrate like fructose. These highly active hydrogenases make *C. necator* an attractive host for microbial electrosynthesis (MES) systems in which H_2 generated electrocatalytically is used to drive microbial conversion of CO_2 [19–21]. It should be noted that hydrogenotrophic growth is not a general feature of the *C. necator* species; the type strain N-1 lacks the megaplasmid and the hydrogenases it encodes, making it incapable of growth using H_2 [22].

In addition to CO_2, *C. necator* H16 is also capable of growth on formate, another C1 compound. Formate can act as its sole source of carbon and energy via the activity of formate dehydrogenases (FDH) that generate CO_2 and a reducing equivalent that drive the CBB cycle (Figure 2) [16]. Formate/Formic acid is of particular

Figure 2



C1 and energy metabolism in *C. necator*. Hydrogen or formate provides the reductive power to drives the respiratory chain (RC) and enable CO_2 fixation via the CBB cycle. Other abbreviations used in this figure: AS, ATP synthase; G3P, glyceraldehyde-3-phosphate, ND, NADH dehydrogenase; RuBisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase; SH, soluble hydrogenase; TH, pyridine nucleotide transhydrogenase.

interest as it can be generated by electrocatalytic reduction of CO_2, making it an attractive substrate to enable hybrid bio/electrocatalytic systems for CO_2 valorization since its solubility overcomes the challenges associated with safety, storage, transportation, and delivery of gaseous CO_2 and H_2 [23]. Formate is relatively toxic to *C. necator*, but this can be mitigated by pH-stat cultivation on formic acid in which its consumption raises the pH, triggering the addition of more formic acid, thereby enabling rapid growth while preventing alkaline stress and the accumulation of toxic concentrations of formate in the bioreactor [24–26].

Methanol represents another soluble C1 compound that can be generated from CO_2 electrocatalytically and used as a feedstock for bioconversion [27]. *C. necator* N-1 possesses an active methanol dehydrogenase, Mdh2, capable of converting methanol to formaldehyde [28]. Formaldehyde could be converted to formate by various aldehyde dehydrogenases, which are known to be promiscuous enzymes [29]; however, this strain is not capable of growth on methanol [28]. *C. necator* H16 has no annotated methanol dehydrogenases [11]. This difference between *C. necator* strains N-1 and H16, as well as the inability of N-1 to grow autotrophically, suggests substantial differences in these strains, and perhaps other closely related strains of the genera *Cupriavidus* and *Ralstonia* [14], that could be recombined to generate strains optimized toward a particular purpose.

While a large genome like that of *C. necator* H16 can enable metabolic versatility, it can also give rise to inefficiency, particularly when applied to biomanufacturing processes aimed at converting a small number of molecules into a small number of target products. Recent analysis by Jahn et al. suggested that up to 40% of its proteome, by mass, represents proteins that have no known function or are unlikely to be utilized in the condition in which they were expressed [17]. This potential superfluity makes *C. necator* an excellent candidate for adaptive laboratory evolution and genome minimization aimed at honing it toward a particular purpose. The megaplasmid pHG1, for example, contains genes required for denitrification (the only means by which *C. necator* is capable of anaerobic growth) as well as operons encoding the CBB cycle and hydrogenases that enable growth on CO_2 but is otherwise dispensable for growth on most substrates. A recent adaptive laboratory evolution (ALE) study found that deletion of the hydrogenase operons from pHG1, or curing the entire megaplasmid from the strain, improves growth on substrates such as fructose, succinate, and formate [25] perhaps by relieving the burden imposed by expression of the hydrogenases, which Jahn et al. found can account for up to 3% of the proteome by mass [17].

Robust, high cell density growth

Anaerobic microorganisms are logical choices for biomanufacturing of short-chain carbon molecules (C1-C4, such as methane, ethanol, acetate, or butyrate) since those compounds are generated obligately to recycle reducing equivalents during anaerobic metabolism. While aerated processes are often more expensive to operate [30], aerobic bacteria can produce larger molecules such as proteins, lipids, oils, fats, and biopolymers without generating anaerobic co-products by using O_2 as the terminal electron acceptor, producing larger amounts of cellular energy to support anabolism and rapid growth; *C. necator* is capable of growth rates up to $0.34\ h^{-1}$ on organic acids, $0.32\ h^{-1}$ on CO_2/H_2, and $0.18\ h^{-1}$ on formate [24]. ALE was used to increase the growth rate on formate to $0.21\ h^{-1}$ and $0.25\ h^{-1}$ in two separate reports, both of which identified loss of pHG1 as beneficial in this condition [25,31].

While conversion rates affect the productivity of a biomanufacturing process, the cell density of a culture can profoundly affect the volumetric productivity and, consequently, its capital and operating expenses. *C. necator* is capable of growth to extraordinarily high cell densities, with 281 g/l CDW for NCIMB 11599 grown on glucose [32] and 91 g/l CDW for ATCC 17697 grown on CO_2/H_2 (in just 40 hours) [33] having been reported in public literature, metrics that are likely lower than those achieved in optimized industrial processes. For context, the highest cell density reported for *E. coli* is 190 g/l, near the limit of fluidity, which is estimated to be between 200 and 250 g/l [34].

In addition to enabling high productivity, high cell density growth naturally lends itself to production of molecules the host can synthesize naturally. In the case of *C. necator*, this includes PHAs, such as PHB, and protein. Several studies have demonstrated its conversion of CO_2/H_2 to PHB at high titers and rates [35]; using strain ATCC 17697, one group achieved a rate of 1.55 g/l/h and titer of 61.9 g/l, which accounted for 67.7% of the CDW, in a continuous stirred tank reactor [33] and 56.4 g/l PHB, 81.4% of the CDW, at 1.06 g/l/h in an airlift bioreactor amended with 0.05% carboxymethylcellulose to increase the viscosity of the culture medium and, subsequently, the oxygen transfer rate [36]. The protein content of *C. necator* biomass can account for up to 70% of the CDW and has an amino acid profile comparable to that of whey, soy, and casein [37], making it an excellent candidate for single-cell protein (SCP) production [38]. As far back as 1967, NASA envisioned that consumption of *C. necator* (re)generated from $H_2/CO_2/O_2$ could supply half of a person's caloric requirements as part of a closed-loop system to support life during long-term space missions [39] and companies such as Lanzatech [40] are currently pursing *C. necator* as a host for conversion of $H_2/CO_2/O_2$ to protein for human consumption. Such

systems have the potential to dramatically reduce the land, water, and energy required for protein production relative to animal or plant sources [41].

Amenability to genetic manipulation

Transfer of plasmid DNA into *C. necator* H16 by conjugation was first described in the late 1980s [42], followed by electroporation in 1995 [43]. Along with the sequencing of its genome, reported first in 2006 [12] and again in 2019 using updated sequencing technology [13], this enabled genetic characterization and development of *C. necator* as a host for heterologous gene expression. These efforts were hampered by low transformation efficiency until Tee et al. described an improved electroporation method in 2017 [44] and, soon after, Xiong et al. demonstrated that deletion of the restriction enzyme gene H16_A0006 improved transformation efficiency from ~25 colony-forming units/ug DNA to >40 000, a factor of 1658 [45]. This allowed them to demonstrate, for the first time, CRISPR-Cas9 genome editing in *C. necator*. A recent investigation aimed at further improving transformation efficiency in *C. necator* found that combining the transformation methods described by Tee et al. with deletion of the restriction enzyme genes yielded an impressive $>10^7$ colony-forming units (CFU)/ug of DNA [46]. Improved transformation efficiency has enabled the development of additional genetic tools and metabolic engineering strategies [47]. Coupled with its robust autotropic growth, *C. necator's* genetic amenability has enabled it to be engineered to produce several products directly from C1 compounds (Table 1).

Despite the fact that the CBB cycle has fixed the vast majority of inorganic carbon on earth [68], it is often noted as being nonspecific (in additional to carboxylation, it also catalyzes nonproductive oxygenation) and slow [69], making it a suboptimal pathway for biomanufacturing. However, a recent computational analysis considering thermodynamic and kinetic parameters showed that, despite the lower stoichiometric energy efficiency, the CBB cycle is much more active than previously assumed [70]. In particular, fitting kinetic data from *C. necator* in a chemolithotrophic scenario showed that the CBB could achieve product-to-substrate yields comparable to other C1 fixation pathways. Hence, *C. necator* converting CO_2 via the CBB cycle shows promise for the development of C1-based biomanufacturing processes, and several recent studies have sought to improve this capability. At least two studies have demonstrated that heterologous expression of a cyanobacterial RuBisCOs that exhibits high catalytic carboxylation activity in *C. necator* improved its growth rate and biomass yield on CO_2/H_2 [71,72]. These studies also illustrated that tuning expression of endogenous genes can improve autotrophic growth as well; one found additional benefit from overexpression of the MBH and SH [72], while the other demonstrated further improvement by overexpressing CbbR and RegA, which are transcriptional regulators of the *cbb* operons in *C. necator* [71].

Table 1

Products produced by engineered *C. necator* H16 using C1 feedstocks.

Product	Feedstock	Format	Titer (g/l)	Reference
1,3-Butanediol	$H_2/CO_2/O_2$	Shake flask	2.97 g/l	[48]
3-Hydroxybutanone	$H_2/CO_2/O_2$ 80:5:15	Batch bioreactor	3.9 g/l	[49]
α-Amylase	$H_2/CO_2/O_2$ 75:10:15	Shake flask	4.26 U/OD_{600}	[50]
Crotonate	Formate/formic acid	Fed-batch bioreactor	148 mg/l	[26]
Fatty acids	$H_2/CO_2/O_2$ 7:1:1	Batch bioreactor	60.6 mg/l	[51]
Glucose	$H_2/CO_2/O_2$ 8:1:1	Batch pressurized bottle	240 mg/l	[52]
α-Humulene	$H_2/CO_2/O_2$	MES	10.8 mg/l	[21]
α-Humulene	$H_2/CO_2/O_2$ 77:5:4	Batch bioreactor	146 mg/l	[53]
Isobutanol, 3-methyl-1-butanol	Formic acid	Bioreactor, MES	1.4 g/l, 140 mg/l	[54]
Isopropanol	$H_2/CO_2/O_2$	MES	216 mg/l	[20]
Isopropanol	$H_2/CO_2/O_2/N_2$ 60:10:2:28	Batch bioreactor	3.5 g/l	[55]
L-isoleucine	$H_2/CO_2/O_2$ 8:1:1	Batch pressurized bottle	105 mg/l	[56]
L-valine	$H_2/CO_2/O_2$ 8:1:1	Batch pressurized bottle	319 mg/l	[56]
Lipochitooligosaccharide	$H_2/CO_2/O_2$	Batch pressurized bottle	1.4 mg/l	[57]
Lycopene	$H_2/CO_2/O_2$	MES	1.7 mg/l	[19]
Mannitol	$H_2/CO_2/O_2$	Batch bioreactor	3.9 g/l	[58]
Methyl-ketones	$H_2/CO_2/O_2$ 80:16:4	Batch bioreactor	180 mg/l	[59]
Myo-inositol	$H_2/CO_2/O_2$ 8:1:1	Batch pressurized bottle	1.05 g/l	[60]
N-acetylglucosamine	$H_2/CO_2/O_2$ 8:1:1	Batch pressurized bottle	75.3 mg/l	[61]
N-butanol	Formate/formic acid	Batch tubes	30.0 mg/l	[62]
PHA copolymers	$H_2/CO_2/O_2/N_2$ 3.8:13.0:7.3:75.9	Batch bioreactor	0.20–0.60 g/l	[63]
Phytase	$H_2/CO_2/O_2$	Batch bioreactor	22.3 ± 2.4 U/ml	[64]
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)	$H_2/CO_2/O_2$ 88:10:2	Batch bioreactor	0.65 g/l	[65]
Resveratrol	$H_2/CO_2/O_2$ 88:10:2	Batch bioreactor	1.9 mg/l	[66]
Trehalose	$H_2/CO_2/O_2$ 6:1:1	Batch pressurized bottle	470 mg/l	[67]

It is worth noting that *C. necator* is capable of growth on CO_2 at concentrations found in air [73,74], but the high O_2/CO_2 ratio causes RuBisCO to catalyze oxygenation more frequently than at lower ratios. Oxygenation results in the production of 2-phosphoglycerate that must be salvaged in an unproductive cycle that was recently elucidated in *C. necator* [74]. Cyanobacteria and some chemolithoautotrophs fix CO_2 from ambient air more efficiently by concentrating CO_2 near RuBioCO using CO_2-concentrating Mechanisms (CCMs). These CCMs include transporters that enable bicarbonate (HCO_3^-) to pass across the membrane and carbonic anhydrases (CA) that interconvert HCO_3^- and CO_2. Recent studies have aimed to improve CO_2 fixation in *C. necator* by introducing heterologous bicarbonate HCO_3^- transporters and/or CA [73,75,76]. One study demonstrated higher biomass accumulation in strains expressing a HCO_3^- transporter or a heterologous CA, in place of the two main endogenous ones, in batch cultures grown on 1.5% or 5% CO_2 [75]. A more recent study demonstrated similar improvements using strains expressing HCO_3^- transporters in batch cultures but not when the same strains were cultivated in flow bioreactors [73]. Still, these results show promise toward implementing CCMs that might improve CO_2 fixation in *C. necator*.

As discussed above, *C. necator* is capable of growing on formic acid/formate, which can be generated by electrocatalytic reduction of CO_2. Recent studies have demonstrated that *C. necator* can be engineered to produce various products using formic acid/formate as the sole source of carbon and energy (Table 1). Metabolic engineering has also been useful toward improving formate assimilation. Introduction of a heterologous phosphoketolase, which enables the conversion of CBB intermediates to acetyl-CoA while avoiding the otherwise obviate loss of CO_2 generated by decarboxylation of pyruvate was shown to increase the biomass yield of *C. necator* on formate by 30% [77]. ALE-inspired metabolic evolution demonstrated that growth of *C. necator* on formate can be improved by deleting the copy of the *cbb* operon encoded on the pHG1 megaplasmid [25]. While this may seem paradoxical since formate is ultimately consumed via the CBB cycle, transcriptomics revealed that this deletion resulted in increased expression of the paralogous *cbb* operon on chromosome 2. This study also demonstrated improved growth on formate by deleting the hydrogenases or pHG1 entirely (as described above) or the gene encoding the transcription factor PhcA, likely by reducing expression of genes important for motility, which is energetically costly but dispensable in a bioreactor. Improvement of growth rates on formate upon loss of pHG1 was recently described in a separate ALE study [31].

Despite enabling robust growth of *C. necator* on CO_2 and formate, the CBB cycle consumes more energy equivalents compared to other carbon fixation pathways [78]. Hence, in recent years, several metabolic engineering campaigns have aimed to engineer more efficient pathways and/or introduce them into organisms not capable of carbon fixation [79]. One promising alternative carbon fixation pathway, the reductive glycine pathway (rGP), was recently introduced in *C. necator* to substitute the CBB cycle [80]. After several rounds of engineering and adaptive laboratory evolution, the authors demonstrated growth of *C. necator* with the rGP solely on formate at biomass yields comparable to a strain utilizing the CBB cycle, although at lower growth rates. After further rounds of engineering and adaptive evolution, the same research group demonstrated that the rGP strain could reach yields 17% higher than the WT utilizing the CBB [81]. While this strain still exhibited lower growth rates than the WT strain, this study reflects the great promise of synthetic biology to overcome limitations inherent to natural systems.

Carbon monoxide (CO) is another C1 compound that can be generated by reduction of CO_2 and is also present in many industrial waste gases that can serve as feedstocks for biological conversion. *C. necator* is natively unable to metabolize CO but heterologous expression or extracellular presentation of a CO dehydrogenase has been shown to confer *C. necator* with this ability [82,83]. *C. necator* is remarkably tolerant to CO, and this quality was further enhanced using ALE [84]. These studies suggest *C. necator* could be a promising host for engineering and/or evolution aimed at enabling CO conversion.

Conclusion

The abatement of greenhouse gases on the scale required to curb global warming will require the development and deployment of diverse technologies that optimize the utilization of variable and disparate carbon and energy sources. Aerobic conversion of C1 molecules in processes powered by renewable energy represents one possible approach and *C. necator*'s metabolic versatility, robust growth, and genetic amenability make it one of the most promising organisms to enable such systems. Decades of study and industrial cultivation have positioned this organism to contribute substantiallyto a fully realized circular bioeconomy where fuels, chemicals, materials, and foods are biomanufactured primarily from C1 compounds with minimal waste.

Author contributions

Stefano Donati: Conceptualization, Writing – original draft, Writing – review & editing.

Christopher W. Johnson: Conceptualization, Writing – original draft, Writing – review & editing.

Data Availability

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

Declaration of Competing Interest

C.W.J. has submitted a patent application on engineered strains referenced in this work.

Acknowledgements

We thank Justin Panich, Adam M. Guss, Gregg Beckham, and Enrico Orsi for their valuable feedback. This work was authored in part by the National Renewable Energy Laboratory for the U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. Funding provided by U.S. Department of Energy Office of Energy Efficiency and Renewable Energy Bioenergy Technologies Office and Office of Science Biological and Environmental Research Program. The views expressed in the article do not necessarily represent the views of the DOE or the U.S. Government. The U.S. Government retains and the publisher, by accepting the article for publication, acknowledges that the U.S. Government retains a non-exclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this work, or allow others to do so, for U.S. Government purposes. S.D. acknowledges support from the Novo Nordisk Foundation (NNF20CC0035580).

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- • of special interest
- •• of outstanding interest

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