



Review

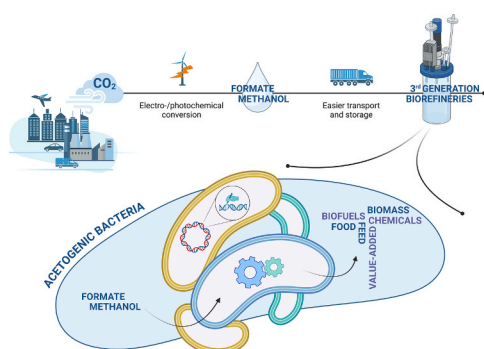
Utilization of the liquid one carbon feedstocks methanol and formate for acetogenic bioproduction of chemicals and fuels

Eugenio Pettinato, Rémi Hocq, Stefan Pflügl*

Institute for Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, Gumpendorfer Straße 1a, 1060 Vienna, Austria



GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Formatotrophy
Methylotrophy
Acetogens
Metabolic engineering
Bioenergetics
Sustainable bioprocessing
Circular carbon economy

ABSTRACT

The fight against climate change requires consideration of carbon as a critical parameter in production systems, with the ultimate aim of creating a truly sustainable circular carbon economy. In this context, microbial bio-production systems are a promising route to renewably generate value-added chemicals and fuels. Methanol and formate have recently gained interest as microbial one-carbon feedstocks, which can be produced sustainably from carbon dioxide and renewable energy, are easy to store and transport and readily dissolve in aqueous solutions. Acetogenic bacteria are strictly anaerobic microorganisms that can grow autotrophically on molecular hydrogen or use methanol, formate, and carbon monoxide as their sole carbon and energy sources via the Wood-Ljungdahl pathway, the most energetically efficient carbon fixation pathway known to date. Here, known variants of the Wood-Ljungdahl pathway, the physiology of a selection of methylotrophic and formatotrophic acetogens, and emphasize recent advancements in bioprocessing with respect to quantification of acetogen metabolism of methanol and formate as well as research aiming at establishing novel bioprocesses are reviewed. Additionally, the tools available for physiological and metabolic studies as well as for metabolic and genetic engineering are discussed. Finally, the features and constraints that govern the bioenergetics and stoichiometry of acetogen metabolism during growth on methanol and formate are reviewed, and future perspectives of the field discussed. The high energetic efficiency with which acetogens can convert methanol and formate into products renders them highly attractive platform hosts in the circular carbon economy.

* Corresponding author.

E-mail address: stefan.pfluegl@tuwien.ac.at (S. Pflügl).<https://doi.org/10.1016/j.biortech.2025.132643>

Received 28 March 2025; Received in revised form 30 April 2025; Accepted 7 May 2025

Available online 16 May 2025

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1. Introduction

The rise of atmospheric carbon dioxide (CO₂) concentrations is a direct cause of global warming, and has been shown to be driven primarily by the extensive use of fossil fuels to meet the energy and material demands of human society (International Energy Agency, 2023; Levi and Cullen, 2018; Vom Berg et al., 2022). The principles of circularity and sustainability applied to the carbon economy suggest that it is crucial not only to reduce emissions, but also to find ways to harness, store and reuse carbon, placing it at the center of cyclical production lines for the supply of raw materials and fuels (Alsarhan et al., 2021). Industrial biotechnology can help by providing alternative methods using microbes and fermentation technologies as platforms for the bio-based production of chemicals, ideally adding value to waste products from other sectors (Bachleitner et al., 2023; Lee et al., 2023).

One-carbon (C1) substrates and, in general, gas mixtures containing different inorganic compounds (e.g., methane, carbon monoxide and hydrogen/CO₂) have recently gained attention as alternative feedstocks for microbial growth, giving rise to so-called third-generation biorefineries (Liu et al., 2020). Industrially attractive gas-fermenting organisms typically belong to the *Clostridia* class (e.g. *Acetobacterium*, *Moorella*, *Clostridium*, *Eubacterium*, *Thermoanaerobacter* genera), and the case of “*Clostridium autoethanogenum*”, used on an industrial scale by LanzaTech for the conversion of carbon monoxide (CO) to bioethanol, represents a successful example of this development (Cotton et al., 2020; Köpke and Simpson, 2020). Substrates to be used as feed in third-generation biorefineries can be derived from a variety of diffuse and point sources, including the natural environment (Strong et al., 2015), industrial waste gases (Dürre and Eikmanns, 2015), gasification of biomass or solid waste (i.e. syngas) (Mauerhofer et al., 2021), or even via direct CO₂ capture from the atmosphere (Cheah et al., 2016).

However, there are a number of technical limitations to the use of gas mixtures in industrial fermentation. Achieving high microbial productivity in a gas fermentation process requires high gas transfer rates into the liquid phase of the fermentation, which depends both on the driving force (mainly affected by the low gas solubility) and the gas–liquid mass transfer coefficient (k_ga) (Liew et al., 2016; Puiman et al., 2022). Furthermore, the ability to safely store, transport and generally handle gases is an additional challenge in the context of their industrial use. In contrast, liquid C1 substrates such as methanol and formate do not present the same problems, as they are fully miscible and can also be easily transported and stored, allowing for a decoupling of feedstock production and consumption (Cotton et al., 2020).

Methanol is now an important building block in the chemical industry (Kang et al., 2021), but has also been proposed as a key feedstock in a “methanol economy” by Nobel Laureate George Olah to overcome the use of fossil fuels (Olah et al., 2018). In the context of economic models focusing on biological resources and technologies (i.e., bio-economy), the potential of methanol as a microbial feedstock has been discussed for many decades (Dijkhuizen et al., 1985; Schrader et al., 2009). Formate, on the other hand, has received less attention due to its relatively high cost, and only a few studies have considered it as a main substrate for microbial fermentation (Dronsella et al., 2025; Kim et al., 2020; Nattermann et al., 2023). However, as recent technological developments have made it possible to synthesize both compounds electrochemically or photochemically from CO₂ and renewable energy, (Ewis et al., 2023; Li et al., 2022), the design of a bioeconomy rooted in methanol and formate as microbial feedstocks has gained momentum (Olah et al., 2018; Yishai et al., 2016).

Of the seven natural autotrophic carbon fixation pathways known to date, the Wood-Ljungdahl pathway (WLP) is the most energetically efficient and allows the conversion of not only CO₂ but also other C1 substrates (e.g., CO, formate, methanol) into biomass and value-added products (e.g., acetate) (Ljungdahl, 1986; Wood, 1991). This WLP functions in strict anaerobes living at the thermodynamic limit of life, such as methanogenic archaea and acetogenic bacteria (Ragsdale, 2008;

Thauer et al., 2008). However, the high energetic efficiency of the WLP comes at the cost of an overall low ATP output, limiting the product spectrum in acetogens (Schuchmann and Müller, 2014).

This review discusses how the WLP enables methylotrophic and formatotrophic growth, highlighting the various enzymes, co-factors and electron carriers identified in the currently known variants in acetogenic bacteria. Subsequently, a selection of industrially attractive acetogens is presented outlining their physiology. Next, recent achievements in bioengineering aiming at establishing novel bioprocesses using methanol and formate as feedstocks and the acetogens in question as biocatalysts are highlighted. To that end, the available genetic and analytical tools that have been developed to study the metabolism of acetogens at a systems level and/or to extend their product range to non-natural metabolites are reviewed. The bioenergetic landscape of acetogen metabolism is then analyzed, with particular emphasis on the stoichiometry and limitations governing methylotrophic and formatotrophic growth. Finally, future directions in the field are explored, highlighting the potential of acetogens to transform industrial bioproduction through their remarkable energetic efficiency in converting methanol and formate into a product.

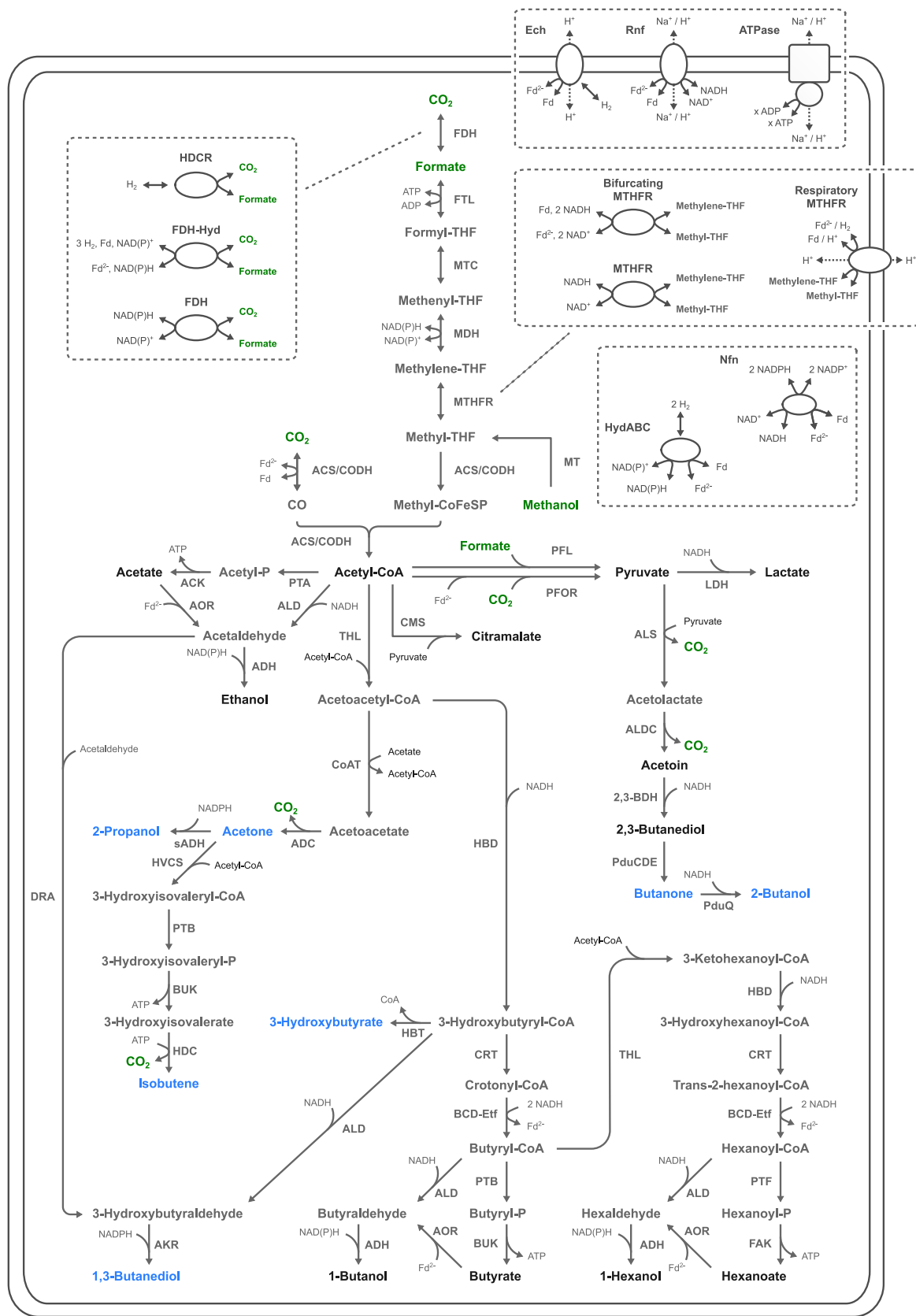
2. Variants of the Wood-Ljungdahl pathway functioning in acetogenic bacteria

The metabolic intermediates of the WLP were established in the 1980s and are conserved across all acetogens (Ragsdale and Pierce, 2008; Schuchmann and Müller, 2014). However, there are differences among acetogens in some of the enzymes of the WLP, which dictate the specific redox co-factors required to drive this pathway in a given microbe. In addition to the widespread redox equivalents NADH and NADPH, acetogens notably exploit a low potential electron carrier, ferredoxin (Fd or Fd²⁻, depending on the redox state), to drive critical enzymatic reactions, such as the reduction of CO₂ to carbon monoxide (CO). Fig. 1 shows the WLP, its enzymatic variants and related redox co-factors, the entry points for both methanol and formate, and a selection of product pathways used to model the bioenergetics during growth on methanol/formate (see section 5 Bioenergetics).

The WLP consists of two branches that lead to the condensation of two molecules of CO₂ to acetyl-CoA: the carbonyl and the methyl branch. The first step of the methyl branch reduces CO₂ to formate, as a result of the activity of an NAD(P)H-dependent formate dehydrogenase (FDH) [e.g., in *Moorella thermoacetica* (Andresen and Ljungdahl, 1974)], a formate dehydrogenase associated with an electron-bifurcating hydrogenase (FDH-Hyd) which couples CO₂, oxidized ferredoxin and NAD(P)⁺ reduction to molecular hydrogen (H₂) oxidation [e.g., in *Eubacterium limosum* (Dietrich et al., 2021)], or the hydrogen-dependent CO₂ reductase (HDCCR) [e.g., in *Acetobacterium woodii*, (Schuchmann and Müller, 2013)].

The methyl branch proceeds with the ATP-dependent activation of formate with tetrahydrofolate (THF) by formate-THF ligase (FTL) and then with the formation of methenyl-THF by the activity of methenyl-THF cyclohydrolase (MTC). Methenyl-THF is reduced to methylene-THF by methylene-THF dehydrogenase (MDH), which is either NADH or NADPH dependent, depending on the acetogen (Katsyv et al., 2021; Ragsdale and Ljungdahl, 1984).

The next step is the reduction to methyl-THF, which is catalyzed by methylene-THF reductases (MTHFRs), a heterogeneous group of enzymes. Four different types of MTHFR have been previously classified (Kremp et al., 2022). Two of them feature cytoplasmic NADH-dependent enzymes, e.g., in *A. woodii* (Bertsch et al., 2015). Fd²⁻-dependent MTHFRs represent another group that remains partly enigmatic, as direct reduction of methylene-THF with Fd²⁻ is not compatible with growth due to the negative ATP yield from H₂/CO₂. It has therefore been proposed that Fd²⁻-dependent MTHFRs are electron-bifurcating, coupling the electron transfer from Fd²⁻ to methylene-THF with either the reduction of another electron carrier (Dietrich et al., 2021), or with



(caption on next page)

Fig. 1. Metabolic pathways linking methanol (+ CO₂) and formate, methanol to a selection of natural and synthetic products in methylotrophic and formatotrophic acetogens. THF: tetrahydrofolate. Fd: ferredoxin. Ech: energy-conserving hydrogenase complex. Rnf: *Rhodobacter* nitrogen fixation complex. FDH: formate dehydrogenase. HDCR: hydrogen-dependent CO₂ reductase. FDH-Hyd: formate dehydrogenase / electron-bifurcating hydrogenase complex. FTL: formyl-THF ligase. MTC: methenyl-THF cyclohydrolase. MDH: methylene-THF dehydrogenase. MTHFR: methylene-THF reductase. MT: methyltransferase. ACS/CODH: Acetyl-CoA synthase / CO dehydrogenase complex. HydABC: electron bifurcating hydrogenase. Nfn: NADH-dependent reduced ferredoxin:NADP + oxidoreductase. PTA: phosphotransacetylase. ACK: acetate kinase. AOR: aldehyde:ferredoxin oxidoreductase. ALD: aldehyde dehydrogenase. ADH: alcohol dehydrogenase. PFL: pyruvate formate lyase. PFOR: pyruvate ferredoxin oxidoreductase. LDH: lactate dehydrogenase. CMS: citramalate synthase. ALS: acetolactate synthase. ALDC: acetolactate decarboxylase. 2,3-BDH: 2,3-butanediol dehydrogenase. PduCDE: B12-dependent propanediol dehydratase. PduQ: secondary alcohol dehydrogenase. THL: thiolase. CoAT: CoA transferase. ADC: acetoacetate decarboxylase. sADH: secondary alcohol dehydrogenase. HVCS: 3-hydroxyisovalerate-CoA synthase. PTB: phosphotransbutyrylase. BUK: butyrate kinase. HDC: 3-hydroxyisovalerate decarboxylase. DRA: 2-deoxyribose-5-phosphate aldolase. AKR: aldo-keto reductase. HBD: 3-hydroxybutyryl-CoA dehydrogenase. HBT: 3-hydroxybutyryl-CoA thioesterase. CRT: crotonase. BCD-Etf: butyryl-CoA dehydrogenase / electron transferring flavoprotein. PTF: phosphotransferase. FAK: fatty acid kinase. Products not naturally produced by methylotrophic and formatotrophic acetogens are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

anaerobic respiration (Katsyv et al., 2021; Öppinger et al., 2021). The last, membrane-bound MTHFR type, reduces methylene-THF with molecular hydrogen (Kremp et al., 2022). In *Sporomusa ovata*, this reaction involves quinones and results in the export of protons across the membrane. The resulting proton gradient subsequently drives ATP formation, and this type of MTHFR is therefore tightly linked with anaerobic respiration. Fig. 1 shows a simplified classification of MTHFRs with three categories: NAD(P)H-dependent, electron-bifurcating, and respiratory-linked enzymes.

The carbonyl branch is mediated by the CO dehydrogenase activity of the acetyl-CoA synthase (ACS/CODH), which captures and reduces one CO₂ into CO with Fd²⁻. The methyl group from methyl-THF is transferred by ACS to a corrinoid iron-sulphur protein carrier (CO-FeSP). Finally, ACS generates acetyl-CoA with CO as the carbonyl donor, methyl-COFeSP as the methyl donor, and CoA as the co-factor.

The production of one acetyl-CoA via the WLP requires one ATP in acetogenic bacteria. The typical major end product of acetogenesis is acetate, which is formed from acetyl-CoA in a reaction that generates one ATP through substrate-level phosphorylation. As a result, the formation of acetate from CO₂ via the WLP is ATP neutral. Net ATP production is only possible via a transmembrane, chemiosmotic mechanism, that couples the Fd²⁻-dependent generation of a gradient of H⁺ (e.g., in “*C. autoethanogenum*”, *M. thermoacetica*, *Thermoanaerobacter kivui*) or Na⁺ (e.g., in *A. woodii*, *E. limosum*) to ATP synthesis (Poehlein et al., 2012; Schuchmann and Müller, 2014).

There are currently two known anaerobic respiratory complexes in acetogens: the *Rhodobacter* nitrogen fixation (Rnf) and the energy-converting hydrogenase (Ech) (Schoelmerich and Müller, 2019; Westphal et al., 2018). Rnf- and Ech-type complexes use the excess energy from the Fd²⁻-mediated reduction of NAD⁺ (e.g., in *A. woodii*, *E. limosum*, “*C. autoethanogenum*”) or H⁺ (e.g., in *M. thermoacetica*, *T. kivui*), respectively, to drive the translocation of H⁺/Na⁺ across the membrane. The required Fd²⁻ is generated from H₂ via an electron-bifurcating hydrogenase, which transfers the electrons from two H₂ to Fd and to NAD(P)⁺.

Collectively, the overall ATP production of acetogen metabolism is determined by the interplay of the enzymes and complexes involved in the WLP and energy metabolism and their co-factor specificity to balance cellular redox pools: H₂, NAD(P)H and reduced ferredoxin (Fd²⁻) by the electron-bifurcating hydrogenase, NADPH, NADH or Fd²⁻ by the ferredoxin-dependent NADH:NADP + oxidoreductase (Nfn or Stn) and the membrane-bound respiratory complexes Rnf and Ech (Fig. 1). Additionally, the type of C1 substrate and the metabolic product affect ATP production (see Section 5 Bioenergetics).

In addition to the ability to grow on H₂/CO₂, some acetogens exhibit methyl- and formatotrophic lifestyles (Table 1). Among these, *A. woodii* and *E. limosum* have arguably emerged as model organisms for formate and methanol utilization (Balch et al., 1977; Kremp et al., 2018; Moon et al., 2021; Neuendorf et al., 2021; Pregnon et al., 2022; Wood et al., 2023a). Formatotrophic growth was first characterized in detail in *A. woodii* only in recent years (Moon et al., 2021; Neuendorf et al., 2021; Novak et al., 2021) (see Section 3 Physiology). During growth on

formate, the downstream reaction to acetyl-CoA in the methyl branch proceeds normally, while the CO₂ moiety needed for the carbonyl branch is provided by the reverse reaction of the formate dehydrogenase. Formate oxidation also provides the electrons needed for energy conservation, ultimately resulting in the production of two molecules of CO₂ per acetate formed. Another potential entry point for formate is via pyruvate formate lyase (PFL), which connects pyruvate and acetyl-CoA. The second option for pyruvate formation in acetogens is via carboxylation of acetyl-CoA at the expense of Fd²⁻ catalyzed by pyruvate:ferredoxin oxidoreductase (PFOR). Both PFL and PFOR have been shown to be fully reversible, with the former operating independent and the latter dependent on the CO₂ concentration. PFOR appears to be the dominant enzyme in acetogen metabolism but there have been reports that PFL is up-regulated during formatotrophic growth in both *A. woodii* and *E. limosum* (Moon et al., 2021; Neuendorf et al., 2021; Wood et al., 2023a). The advantage of PFL over PFOR is that no Fd²⁻ investment is needed for pyruvate synthesis, which has been determined to be a limiting factor during autotrophic growth of acetogens (Heffernan et al., 2020). Consequently, PFL could be exploited to improve bioenergetics in acetogens (see section 5 Bioenergetics).

In *A. woodii* and *E. limosum*, methanol enters the WLP at the level of the methyl-THF (Kremp et al., 2018; Pregnon et al., 2022; Wood et al., 2023b). The methyl group of methanol is transferred to THF by a methyltransferase system (Kremp and Müller, 2021). This system consists of two methyltransferases, MTI and MTII, and a corrinoid protein, CoP, which acts as an intermediate methyl group acceptor (Das et al., 2007; Kremp and Müller, 2021). Methyl-THF produced by the methyltransferase system is subsequently either converted by the ACS or oxidized through reverse operation of the methyl-branch of the WLP, thus generating reduced co-factors necessary for growth. As methanol is a highly reduced substrate, an electron sink such as formate or CO₂ (Kremp et al., 2018; Litty et al., 2022; Wood et al., 2023b) is required to enable methylotrophic growth in acetate-producing acetogens.

The WLP has been envisioned as a metabolic module that could theoretically be “plugged in” to fix CO₂ (Schuchmann and Müller, 2014). The same is true for the methanol assimilation route, which is genetically encoded as a minimal set of genes (Kremp and Müller, 2021; Schuchmann and Müller, 2014), that is, an operon encoding all enzymatic components of the methyltransferase system as well as a transcriptional regulator. Similar to the metabolic modules responsible for substrate assimilation, the product pathways can also be implemented by means of metabolic engineering, thereby expanding the product portfolio of acetogens to other value-added chemicals, provided that the bioenergetics support product formation with high yield (see Section 5 Bioenergetics).

3. Physiology and bioprocess engineering of selected acetogens

Schuchmann and Müller have comprehensively reviewed the autotrophic and heterotrophic metabolisms as well as the bioenergetics of acetogens (Schuchmann and Müller, 2016, 2014). Here, the physiology of selected industrially relevant formatotrophic and methylotrophic

Table 1
Physiological properties of selected acetogens and products obtained during methyl- and/or formatotrophic growth.

Species	Growth temperatures (T _{opt}) [°C]	Feedstocks (specific growth rate, μ)	Defined medium	Product		References
				Natural	Engineered	
<i>Acetobacterium woodii</i>	(30) ¹	Methanol (0.05 h ⁻¹) ² Formate (0.12 h ⁻¹) ³	Yes, with pantothenate ¹ and biotin [#]	Acetate ¹ Formate ³ Ethanol ⁴	Acetone ⁵ Isopropanol ⁶ Lactate ⁷ Butyrate ⁸ Homoformatogenesis ⁹	¹ Balch et al., 1977 ² Krempl et al., 2018 ³ Moon et al., 2021 ⁴ Buschhorn et al., 1989 ⁵ Hoffmeister et al., 2016 ⁶ Arslan et al., 2022 ⁷ Mook et al., 2022a ⁸ Chowdhury et al., 2022 ⁹ Moon et al., 2023
<i>Eubacterium limosum</i>	30–45 (37) ¹	Methanol (0.067–0.076 h ⁻¹) ^{1,2} Formate ³ Methanol + Formate (0.19 h ⁻¹) ⁴	Yes, with pantothenate, biotin and lipoic acid ^{1,4}	Acetate ^{2,4} Butyrate ^{2,4} Hexanoate ^{2,4} Butanol ^{4,5}	Butanol ⁵ Acetone ⁵ Acetoin ⁶ 2,3-butanediol ⁷	¹ Pregnon et al., 2022 ² Flaiz et al., 2024 ³ Wood et al., 2023a ⁴ Wood et al., 2021 ⁵ Flaiz et al., 2021 ⁶ Kang et al., 2020 ⁷ Jin et al., 2022
<i>Eubacterium callanderi</i>	25–45 (37) ^{1,2}	Methanol (0.067 h ⁻¹) ¹ Formate ³	Yes, with pantothenate and biotin ²	Acetate ⁴ Ethanol ⁴ Lactate ⁴ Butyrate ⁴ Butanol ⁴ Hexanoate ⁴	Butanol ⁴ (increased)	¹ Flaiz et al., 2024 ² Chang et al., 1999 ³ Kerby and Zeikus, 1987 ⁴ Humphreys et al., 2022
<i>Eubacteriaceae strain ES2</i>	15–45 (30–35)	Methanol (0.08 h ⁻¹) Formate (0.34 h ⁻¹)	Not tested	Acetate	NA	Yu et al., 2023
<i>Sporomusa ovata</i>	15–45 (34–39) ¹	Methanol (0.05 h ⁻¹) ² Formate ¹ Electrotrophy ³	Yes ¹	Acetate ¹ Ethanol ¹	NA	¹ Möller et al., 1984 ² Tremblay et al., 2015 ³ Madjarov et al., 2022
<i>Clostridium luticellarii</i>	(37)	Methanol	Yes	Isobutyrate Butyrate Caproate Valerate Isocarboxylic acids	NA	Petrognani et al., 2020
<i>Moorella thermoacetica</i>	45–65 (55–60) ¹	Methanol ² Formate ²	Yes ³	Acetate ¹ Ethanol ⁴ Lactate ⁵	Lactate ⁶ Acetone ⁷ Isopropanol ⁸ Acetoin ⁹	¹ Fontaine et al., 1942 ² Redl et al., 2020 ³ Daniel et al., 1990 ⁴ Jia et al., 2023 ⁵ Drake and Daniel, 2004 ⁶ Iwasaki et al., 2017 ⁷ Kato et al., 2021 ⁸ Kato et al., 2024b ⁹ Kato et al., 2024a
<i>Thermoanaerobacter kivui</i>	50–72 (66) ¹	Formate ¹	Yes, without vitamins ¹	Acetate ¹ H ₂	NA	¹ Leigh et al., 1981 ² Burger et al., 2022

NA: not applicable.

#: unpublished observation

acetogens is discussed, and their key characteristics are summarized in Table 1. Specifically, *A. woodii* and *Eubacterium* spp. are discussed, as these microbes have arguably emerged as acetogenic “models” for liquid C1 upgrading. Additionally, recent bioprocess engineering advances in formate and methanol conversion are reviewed.

3.1. *Acetobacterium woodii*

The bioenergetics of the WLP were first elucidated in detail in

A. woodii, a mesophilic, Gram-positive bacterium of the class *Clostridia*, which has accordingly become a model organism for the study of acetogens [type strain: DSM 1030, (Balch et al., 1977; Poehlein et al., 2012; Schmidt et al., 2009)]. *A. woodii* is capable of growing on a considerable range of different organic substrates, with fructose and glucose reported as the preferred sugars (Balch et al., 1977). It can also grow autotrophically with molecular hydrogen (H₂) as electron donor by reducing two molecules of CO₂ to acetyl-CoA, and assimilate various C1 substrates via the WLP (see section 2 Variants of the Wood-Ljungdahl

pathway). These remarkable metabolic characteristics allow *A. woodii* to grow on mineral medium without the addition of yeast extract or vitamins other than pantothenate - an observation already made by the authors of the original publication (Balch et al., 1977) - and biotin (unpublished observation). The main end product of *A. woodii* fermentative metabolism is acetate (homoacetogenesis), but ethanol and formate can also be produced to a lesser extent and only under certain growth conditions (Buschhorn et al., 1989; Moon et al., 2021; Moon and Müller, 2021).

The growth of *A. woodii* on formate as the sole carbon and energy source was demonstrated in 1977 at the time of its first isolation with cultivation studies in serum flasks. Final cell densities of 0.4 (OD₆₆₀) were reported for cultures grown on 100 mM formate (Balch et al., 1977). Soon after, in 1981, the ability of *A. woodii* to grow on methanol was also demonstrated, with final cell densities of 0.1 (OD₅₇₈) reported for cultures supplemented with 5 mM methanol and 0.05 % yeast extract (Bache and Pfennig, 1981). Fundamental aspects of the biochemical, physiological, and genetic background of methyl- and formatotrophic metabolism of *A. woodii* have been investigated extensively by the following studies of the Müller group (Kremp et al., 2018; Litty et al., 2022; Moon et al., 2024; 2021).

Final cell densities of 0.6 and 1.5 (OD₆₀₀) were reported for cultures supplemented with 100–200 mM formate and 60 mM methanol, respectively (Kremp et al., 2018; Moon et al., 2021). Transcriptome analyses shed light on the genome organization and regulation of key genes involved during growth on methanol and formate (Kremp et al., 2018; Moon et al., 2021). In particular, the authors investigated the role of the formate transporter FdhC, the two formyl-THF synthetase isoenzymes, Fhs1 and Fhs2, and HDCR (Moon et al., 2024; 2021). The ability of the $\Delta fhs2$ and $\Delta fhs2/fdhC$ mutants to grow on formate was impaired, highlighting the role of the corresponding genes for formate consumption (Moon et al., 2021).

Transcriptome analysis similarly allowed the identification of key genes involved in methanol conversion in *A. woodii* (Kremp et al., 2018; Litty et al., 2022). The gene cluster Awo_c22810-Awo_c22740 was strongly upregulated in methanol-grown compared to fructose-grown cells (Kremp et al., 2018). Among the eight genes included in the cluster, the methyltransferase system Awo_c22760-Awo_c22740 was also suggested to be present as the main one involved in methanol conversion. This system, composed of methyltransferase I (*mtaB*), corrinoid protein (*mtaC2*) and methyltransferase II (*mtaA*) and was further identified and confirmed as methanol-specific (Litty et al., 2022). Deletion of the gene cluster abolished methylotrophic growth of *A. woodii*. Based on this minimal gene set (*mtaBC2A*) identified for methylotrophic growth of an acetogen, transfer of methylotrophy as a metabolic module to other organisms to establish synthetic, anaerobic methylotrophy might become feasible.

The valuable insights obtained by fundamental studies on acetogen metabolism are typically conducted in serum flasks and are often not followed by cultivation in bioreactors. In fact, in the context of bioprocess engineering, data on *A. woodii* as an acetate production platform under strictly methyl- and formatotrophic growth conditions are not extensive (Neuendorf et al., 2021; Novak et al., 2021). Neuendorf and colleagues focused on the performance parameters and metabolism of *A. woodii* cultivated in bioreactors in continuous mode (i.e., chemostat at a dilution rate of 0.05 h⁻¹) under strictly formatotrophic and mixotrophic growth conditions (i.e., 100 and 200 mM formate; H₂:CO₂; formate/H₂:CO₂; formate/H₂:CO₂:CO; formate/fructose) (Neuendorf et al., 2021). Transcriptomic and proteomic analysis comparing gene and protein expression levels gave an overall picture of *A. woodii* metabolism and confirmed the involvement of key genes also identified by the previously cited studies during formate growth (Moon et al., 2024; 2021). Up-regulation of carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), the formyl-THF synthetase isoenzyme Fhs2, and the formate transporter FdhC was clearly detected in the formate condition (comparatively to the fructose one). During strict

formatotrophic growth (200 mM formate), acetate and biomass titers of 3.17 ± 0.05 and 0.22 ± 0.01 g L⁻¹, respectively, and a formate uptake rate of 47 mmol g⁻¹h⁻¹ were reported (Neuendorf et al., 2021). Additionally, formate supplementation was shown to enable syngas fermentation of *A. woodii* with co-utilization of CO, CO₂ and H₂ (Novak et al., 2021). Furthermore, *A. woodii* demonstrated remarkable metabolic flexibility in mixed feed systems, efficiently co-utilizing formate with fructose, H₂/CO₂, or syngas without exhibiting carbon catabolite repression (Neuendorf et al., 2021). This characteristic highlights its potential as a versatile platform for biotechnological chemical production.

The production of formate, rather than its consumption as a carbon source in the context of C1 substrate valorization, is also an area of research that has gained increasing interest (Moon et al., 2023). Indeed, the reversible production of formate from H₂/CO₂ using *A. woodii* as a platform has been exploited as a solution for the storage of H₂ and the capture of CO₂ (Schwarz et al., 2021). In this scenario, similar to that of syntrophic communities, formic acid assumes the role of a liquid organic hydrogen carrier and acetogenic bacteria, as whole-cell biocatalysts, provide a biological pathway for the targeted conversion of hydrogen to formate and *vice versa*, allowing the decoupling of hydrogen production, storage and reuse. The process was successfully tested for *A. woodii* and *T. kivui* cultivated in batch bioreactors in the following study (Schwarz et al., 2021). To assess the efficiency of the whole-cell biocatalysts in question, the authors reported that the HDCR of *T. kivui* performs CO₂ hydrogenation and formate oxidation with high turnover frequencies (TOF) of up to 9.6 × 10⁶ h⁻¹ and 9.9 × 10⁶ h⁻¹, respectively (Schwarz et al., 2018). These numbers are much higher in comparison to standard chemical catalysts (e.g., palladium) which typically have a TON in the range of ≈10³ (Song et al., 2017; Wang et al., 2024), emphasizing the potential of *A. woodii* for formate production.

3.2. *Eubacterium* spp

The diversity of *Eubacterium* species known today and the unclear consensus on their phylogenetic relationships have raised questions that have been addressed by a recent study that provides a comprehensive overview of their phylogenetic affiliations and physiological characteristics (Flaiz et al., 2024). The genus includes several Gram-positive mesophilic acetogens capable of fermenting C1 substrates, including formate and methanol, to acetate and/or longer-chained organic compounds of high commercial interest (such as butyrate, hexanoate, and butanol, typically synthesized via reverse β -oxidation (Flaiz et al., 2024; Litty and Müller, 2021; Wood et al., 2021). *Eubacterium limosum* (type strain: DSM 20543 / ATCC 8486) is the type species for this genus, which can grow autotrophically with dihydrogen on minimal medium without yeast extract (with pantothenic acid and biotin supplementation) (Chang et al., 1999). The closely related species *E. callanderi* (type strain: DSM 3662) is physiologically highly similar to *E. limosum*. “*Butyribacterium methylotrophicum*” DSM 3468 and “*E. limosum*” KIST612 have now been reclassified as *E. callanderi* strains, and various authors have utilized them as platforms for bioprocess engineering (Bengelsdorf et al., 2016; Flaiz et al., 2024; Humphreys et al., 2022; Kim et al., 2021; Litty and Müller, 2021; Wang et al., 2023; Zeikus et al., 1980).

The metabolic response of *E. limosum* to different concentrations of sodium or different forms of dissolved carbon dioxide were tested during growth with methanol by the following studies (Pacaud et al., 1985; Sharak Genthner and Bryant, 1987). Butyrate production was found to be correlated to the amount of bicarbonate dissolved in the medium (Pacaud et al., 1985). Additionally, *E. limosum* was found to be rather osmotolerant as growth was observed with high sodium (576 mM) or methanol (up to 500 mM) concentrations (Sharak Genthner and Bryant, 1987). Co-feeding *E. limosum* with methanol and glucose resulted in catabolite repression with glucose being the preferred substrate (Sharak Genthner and Bryant, 1987).

Investigating the metabolic response of *E. limosum* in serum bottle and chemostat cultivations provided valuable insights over its methylotrophic and formatotrophic growth abilities (Wood et al., 2023b; a; 2021). *E. limosum* grown under mixotrophic growth conditions with methanol and formate supplemented at different ratios (i. e., 3:1 and 10:1) was shown to prefer formate over methanol. This study reported also the first observed native butanol ($0.5 \pm 0.3 \text{ mmol L}^{-1}$) production in *E. limosum* (Wood et al., 2021). Physiological, proteomics and metabolomics analysis of steady-state chemostat cultivations of *E. limosum* provided performance parameters during continuous cultivations. The uptake rates for formate and methanol were 11.7 ± 2.3 and $26.7 \pm 0.9 \text{ mmol g}_{\text{DCW}}^{-1} \text{ h}^{-1}$, respectively (Wood et al., 2023b; a). During formatotrophic growth, *E. limosum* showed lower formate uptake rates compared to *A. woodii* [i.e., $47 \text{ mmol g}_{\text{DCW}}^{-1} \text{ h}^{-1}$, (Neuendorf et al., 2021)]. However, the methylotrophic growth abilities of *E. limosum* allowed the production of acetate (27 mM) and butyrate (11 mM) and traces of hexanoate as the main fermentation products during methylotrophic growth (Wood et al., 2023b; a; 2021). Co-feeding strategies confirmed that formate is an effective co-substrate (as observed for *A. woodii*) that allows high growth rates, methanol consumption, and in addition triggers “solventogenesis” in *E. limosum* (Wood et al., 2023b).

E. limosum produces an extracellular polymeric substance (EPS) which confers oxygen resistance and enables extracellular electron transfer in electrochemically active bacteria (Dawson et al., 2012; Xiao et al., 2017). This dense biofilm causes different disadvantages for both bioprocessing and genetic engineering applications, and the deletion of the genes involved in EPS biosynthesis resulted in improved handling, processing and engineering availability of the organism (Sanford et al., 2023).

Although *E. callanderi* has not been as thoroughly studied as *A. woodii* or *E. limosum*, the production of longer-chain organic acids has been achieved in several cases. Successful butyrate production (4.3 mM) by *E. callanderi* KIST612 has been achieved in serum bottle batch fermentations during both heterotrophic and strictly methylotrophic growth (Litty and Müller, 2021). The effect of methanol supplementation on syngas-grown cultures of *E. callanderi* in serum bottle batch fermentations was also investigated (Kim et al., 2021). Mixotrophic growth with H_2/CO_2 and methanol had a positive effect on all growth parameters, whereas no significant effect was observed in methanol-supplemented cultures grown with CO/CO_2 as the gas phase (Kim et al., 2021). Similarly, different co-feeding strategies of liquid and gaseous C_1 substrates allowed the maximization of longer-chain organic acids production (i.e., 1.5 mM butanol, 25 mM butyrate, 1.7 mM caproate) in bioreactors by *E. callanderi* DSM 3468 (Humphreys et al., 2022). The results of the study showed higher butyrate titers as well as an improvement in the $\text{C}_4:\text{C}_2$ product ratio (i.e., butyrate:acetate ratio) when the cultures were co-fed with methanol in excess over formate, a trend previously described also for other *Eubacterium* species (Humphreys et al., 2022; Wood et al., 2021). The regulatory mechanisms behind methanol assimilation in *E. callanderi* DSM 3468 have been investigated and methanol assimilation was enhanced in the presence of high lysine concentrations (Wang et al., 2023). In detail, lysine was found to modulate expression levels for formers for nickel (NikABCDE) and cobalamin (FhuBCD), with the former being required by carbon monoxide dehydrogenase and acetyl-CoA synthase and the latter for the methyltransferase system channeling the methyl group into the WLP. The addition of a lysine-rich co-substrate (i.e., corn steep liquor) resulted in an improved conversion of methanol to butyric acid (29 mM) and to acetic acid (37 mM), as well as a 56 % higher final biomass yield by methanol-grown cultures of *E. callanderi* (Wang et al., 2023).

3.3. Other acetogens of interest

Eubacteriaceae sp. ES2 and ES3 convert formate and methanol into acetate very efficiently (Yu et al., 2023). Strain ES2 in particular showed formate tolerance and maximum growth rates under strictly

formatotrophic growth conditions that exceeded those previously reported for *A. woodii* (i.e., 700 versus 500 mM and 0.34 versus 0.12 h^{-1} , respectively) (Moon et al., 2021; Yu et al., 2023). These values make ES2 the most efficient formate-utilizing acetogen known to date.

Sporomusa ovata can metabolize a wide range of organic substrates, including formate and methanol, with acetate as the major fermentation product and ethanol in trace amounts (Möller et al., 1984). In addition, *S. ovata* is also electro-trophic, namely an organism that is capable of using electrons as an energy source directly from electrodes (Hu et al., 2024). These metabolic characteristics make *S. ovata* an ideal candidate for microbial electrosynthesis, a process that couples electron transfer from cathodes and autotrophic CO_2 reduction and/or valorization of C_1 substrates into value-added products (Madjarov et al., 2022; Tremblay et al., 2015).

Petrognani and co-workers isolated *Clostridium luticellarii* from a methylotrophic open culture and demonstrated the ability of this strain to produce isobutyric acid and other organic acids using methanol as the sole carbon and energy source (Petrognani et al., 2020).

M. thermoacetica and *T. kivui* are two model organisms for acetogen metabolism at elevated temperatures. *M. thermoacetica* uses both methanol and formate (Drake and Daniel, 2004), while *T. kivui* is only able of formatotrophic growth (Leigh et al., 1981). The innate capability of these microbes to grow at higher temperature ($50\text{--}70^\circ\text{C}$) render them particularly attractive for bioprocessing, as the use of thermophiles in industrial setups can translate into reduced cooling costs and risks of contamination by other species, both of which can be a significant cost driver at large scale (Sitara et al., 2024).

4. Metabolic engineering and systems level analysis

Developing a sustainable bioprocess based on acetogenic methylotrophs and formatotrophs first requires circumventing limitations inherent to these microbes. Indeed, their ancient metabolism is often associated with limited ATP yields, which severely limits the product range and specificity. This is particularly true for formate metabolism, as acetate production from formate typically yields less than one ATP per acetate formed (~ 0.3 ATP per mol acetate in *A. woodii*) (Moon et al., 2021), which is therefore incompatible with the production of ATP-demanding chemicals and fuels without co-production of acetate (e.g., approximately -0.7 ATP per mol lactate in *A. woodii*) (Bertsch and Müller, 2015). In this context, metabolic engineering can provide the necessary keys to 1) increase methylotrophic and formatotrophic ATP yields and 2) redirect the carbon and electron flow towards economically valuable products other than acetate. However, metabolic engineering has its own prerequisites, as its successful implementation necessitates efficient genetic tools as well as a solid, quantitative understanding of the metabolism of a given microbe.

The genetic tractability of a methylotrophic and/or formatotrophic organism of interest constitutes a potentially significant barrier. However, modern genetic tools have progressively been developed in the recent years. These notably include replicative plasmids, CRISPR-dependent and -independent genome editing systems, fluorescent systems and promoter libraries, and gene knockdown systems (Table 2). Of all methylotrophic and formatotrophic acetogens, *A. woodii*, *E. limosum* and *T. kivui* arguably boast the most advanced genetic toolboxes. They all display high transformation efficiencies ($\approx 10^5$ CFU μg^{-1}) (Baker et al., 2022; Sanford et al., 2023; Sitara et al., 2025), and highly efficient genome editing tools based on CRISPR systems (30–100 % editing efficiency depending on the microbe) (Poulalier-Delavelle et al., 2023; Shin et al., 2019; Sitara et al., 2025). Promoter parts are necessary to fine-tune expression of heterologous pathways, and promoters have been characterized in *T. kivui* (Hocq et al., 2023), *E. limosum* (Sanford and Woolston, 2022; Shin et al., 2019; Song et al., 2022), and *A. woodii* (Beck et al., 2020). Some inducible systems have been established in *A. woodii* and *E. limosum*, with a dynamic range of ≈ 70 -fold and >5 -fold (no signal found without induction), respectively (Beck et al., 2020; Sanford and Woolston, 2022). Taken together, these tools have

Table 2
Genetic tools for metabolic engineering of methyl- and formatotrophic acetogens.

Type	<i>A. woodii</i>	<i>A. wieringae</i>	<i>E. limosum</i>	<i>E. callanderi</i> *	<i>S. ovata</i>	<i>T. kivui</i>	<i>M. thermoacetica</i>	References
Transformation	Electroporation ¹ Conjugation ¹	Electroporation ²	Electroporation ^{3,4,5}	Electroporation ^{6,7,8}	Electroporation ⁹	Natural competence ¹⁰	Electroporation ¹¹	¹ Strätz et al., 1994 ² Moreira et al., 2023 ³ Sanford and Woolston, 2022 ⁴ Song et al., 2018 ⁵ Song et al., 2022a ⁶ Humphreys et al., 2022 ⁷ Jeong et al., 2020 ⁸ Wang et al., 2021 ⁹ Tremblay and Zhang, 2023 ¹⁰ Basen et al., 2018 ¹¹ Kita et al., 2013
Antibiotic resistance markers	Thiamphenicol ¹ Clarithromycin ¹ Tetracyclin ²	Thiamphenicol ³	Thiamphenicol ⁴ Clarithromycin ⁴ Carbenicillin ⁵ Tetracyclin ⁵	Erythromycin ^{6,7} Thiamphenicol ^{7,8}	Erythromycin ⁹ Chloramphenicol ⁹	Kanamycin ¹⁰	Kanamycin ¹¹	¹ Straub et al., 2014 ² Strätz et al., 1994 ³ Moreira et al., 2023 ⁴ Shin et al., 2019 ⁵ Sanford and Woolston, 2022 ⁶ Jeong et al., 2020 ⁷ Wang et al., 2021 ⁸ Humphreys et al., 2022 ⁹ Tremblay and Zhang, 2023 ¹⁰ Basen et al., 2018 ¹¹ Kita et al., 2013
Origin of replication	pAMβ1 ¹ pIP404 ^{2,3} pBP1 ^{3,4} pCB102 ^{3,4} pCD6 ^{3,4}	pBP1 ⁵ pCB102 ⁵ pIM13 ⁵	pIP404 ⁶ pBP1 ⁷ pCB102 ^{6,7}	pIP404 ^{8,9,10} pBP1 ⁹ pCB102 ^{9,10} pCD6 ^{9,10} pIM13 ⁹	pIP404 ¹¹ pCD6 ¹¹	pB6A ¹²	pRKU1 ¹³	¹ Strätz et al., 1994 ² Straub et al., 2014 ³ Hoffmeister et al., 2016 ⁴ Baker et al., 2022 ⁵ Moreira et al., 2023 ⁶ Shin et al., 2019 ⁷ Sanford and Woolston, 2022 ⁸ Jeong et al., 2020 ⁹ X. Wang et al., 2021 ¹⁰ Humphreys et al., 2022 ¹¹ Tremblay and Zhang, 2023 ¹² Basen et al., 2018 ¹³ Bourgade et al., 2022
Characterized promoters	Constitutive ¹ aTc-inducible ¹ Lactose-inducible ¹ Theophylline-inducible ¹	Constitutive ²	Constitutive ^{3,4,5} aTc-inducible ^{3,6} Lactose-inducible ³	Constitutive ⁷	Constitutive ⁸	Constitutive ⁹ Mannitol-inducible** Fructose-inducible**		¹ Beck et al., 2020 ² Moreira et al., 2023 ³ Sanford and Woolston, 2022 ⁴ Song et al., 2022a ⁵ Flaiz et al., 2021 ⁶ Shin et al., 2019 ⁷ Jeong et al., 2020

(continued on next page)

Table 2 (continued)

Type	<i>A. woodii</i>	<i>A. wieringae</i>	<i>E. limosum</i>	<i>E. callanderi</i> *	<i>S. ovata</i>	<i>T. kivui</i>	<i>M. thermoacetica</i>	References
Gene knockdown			CRISPRi ^{1,2}			CRISPRi ³		⁸ Tremblay and Zhang, 2023 ⁹ Hocq et al., 2023 ¹ Shin et al., 2019 ² Shin et al., 2023 ³ Sitara et al., 2025
Fluorescent reporter	FAST ^{1,2,3}		FAST ^{1,4} CreiLOV ⁵ eGFP ⁶ HaloTag ⁶ SNAP-Tag ⁶	FAST ⁷ FbFP ⁸		FAST ⁹		¹ Flaiz et al., 2022 ² Mook et al., 2022a ³ Poulalier-Delavelle et al., 2023 ⁴ Flaiz et al., 2021 ⁵ Shin et al., 2019 ⁶ Song et al., 2022a ⁷ Flaiz et al., 2024 ⁸ Wang et al., 2021 ⁹ Hocq et al., 2023
Enzymatic reporter	β -glucuronidase ¹	β -galactosidase ²	β -galactosidase ³ chloramphenicol acetyltransferase ³	β -glucuronidase ⁴	β -glucuronidase ⁵	β -galactosidase**		¹ Beck et al., 2020 ² Moreira et al., 2023 ³ Sanford and Woolston, 2022 ⁴ Jeong et al., 2020 ⁵ Tremblay and Zhang, 2023 ⁸ Wang et al., 2021 ⁹ Hocq et al., 2023
Genome editing based on autotrophic markers	<i>pyrE</i> -based ^{1,2}				<i>pyrE</i> -based ³	<i>pyrE</i> -based ⁴	<i>pyrF</i> -based ⁵	¹ Baker et al., 2022 ² Westphal et al., 2018 ³ Tremblay and Zhang, 2023 ⁴ Basen et al., 2018 ⁵ Kita et al., 2013
CRISPR-based genome editing	Endogenous type I-B ¹		CRISPR-Cas9 ²	CRISPR-Cas9 ³		Endogenous type I-B ⁴		¹ Poulalier-Delavelle et al., 2023 ² Shin et al., 2019 ³ Jeong et al., 2020 ⁴ Sitara et al., 2025
Other genome editing systems			Toxin-antitoxin-based ¹ Cre-Lox-based Recombineering ^{2,3}					¹ Millard et al., 2023 ² Sanford et al., 2024 ³ Sanford and Woolston, 2024

*Following reclassification by Flaiz and colleagues, *E. callanderi* notably encompasses strains previously known as “*E. limosum* KIST612” and “*Butyrivacterium methylotrophicum* DSM 3468”.

**See the preprint by Zeldes and colleagues: Knock-down of genes essential for homoacetogenic growth using sugar inducible promoters in the thermophile *Thermoanaerobacter kivui*. BioRxiv 2024. <https://doi.org/10.1101/2024.06.18.598388>.

permitted the rational engineering of acetogenic methylotrophs and formatotrophs. See section 3 Physiology and Table 1 for an overview of engineered product pathways successfully implemented in acetogens.

A major issue with developing economically viable bioprocesses based on *A. woodii* is its product range, that is naturally limited to acetate. However, in the age of genome editing, metabolic engineering has the potential to solve that problem, by implementing or disrupting product pathways in the metabolic network of *A. woodii*. Indeed, the product range of *A. woodii* has already been expanded to non-natural compounds, although the corresponding body of work generally focused on other substrates than methanol and formate (e.g., H_2/CO_2). Notably, butyrate production was engineered from methanol and CO [0.25 mM, (Chowdhury et al., 2022)]. Other products include acetone and isopropanol [7.6 and 14.5 mM from H_2/CO_2 (Arslan et al., 2022; Höfele et al., 2023; Hoffmeister et al., 2016)], butyrate and hexanoate [0.69 and 1.56 mM from H_2/CO_2 , respectively (Wirth and Dürre, 2021)] and lactate [18.8 mM from H_2/CO_2 , (Mook et al., 2022)]. Interestingly, lactate-producing *A. woodii* could be further coupled to chain elongation in a synthetic coculture with *Clostridium drakei*, providing an alternative pathway to produce hexanoate from H_2/CO_2 [0.95 mM, (Herzog et al., 2022)], and possibly from other C1 compounds.

In addition to expanding the product spectrum, rational metabolic engineering can be applied to enhance substrate uptake performance. In the context of formate utilization in acetogens, a major milestone was achieved by Moon and coworkers (Moon et al., 2024). Two CO-sensitive hydrogenases were previously deleted [namely, the HydA2 subunit of the HDCR and the electron-bifurcating hydrogenase HydABC (Dietrich et al., 2022; Schuchmann and Müller, 2013; 2012)], and the resulting mutant was adapted to grow on CO over a 6-month period (Moon et al., 2024). In addition to growth on CO, the double deletion mutant $\Delta hydBA/hydA2$ was also able to achieve biomass titers twice that of the wild-type strain on formate, which was hypothesized to stem from switching the HDCR co-factor from H^+ to Fd. Such a change in cofactor specificity is indeed predicted to lead to an increase in ATP yield compared to the wild type. Sequencing showed an additional mutation in the *hycB2* gene of the HDCR, which might improve utilization of Fd as a redox partner. Of note, a similar result could also be obtained in *T. kivui*, in which the deletion of the *hydA2* subunit gene of the HDCR dramatically increased its formatotrophic capability (Dietrich and Müller, 2023). Rationally increasing the bioenergetics from formate or from methanol opens up interesting possibilities for bioproduction, as the resulting modified strains might be used to synthesize energetically-demanding chemicals (e.g., propanol).

Compared to *A. woodii*, *Eubacterium* spp. naturally convert methanol / formate into a wider range of products (e.g., butyrate, butanol). In this context, metabolic engineering has notably been exploited as a tool to increase product specificity towards butanol. Plasmid-based over-expression indeed enabled the conversion of methanol up to 0.6 mM in *E. limosum* (Flaiz et al., 2021) and 1.5 mM from methanol in *E. callanderi* (Humphreys et al., 2022). Additionally, acetone production was engineered in *E. limosum* [1.6 mM from methanol (Flaiz et al., 2021)]. Taken together, these results demonstrate that *Eubacterium* spp. are amenable to metabolic engineering, and underline their potential for targeted bioproduction from C1 moieties, particularly in a case in which targeted genome editing approaches can be harnessed.

For acetogenic methylotrophs and formatotrophs, rational genetic engineering is limited to a few strains (e.g., *A. woodii*, *E. limosum*, *T. kivui*), and considerable efforts remain to be undertaken to develop refined genetic tools for other acetogenic methylotrophs / formatotrophs. In genetically recalcitrant species, adaptive laboratory evolution (ALE) (Dragosits and Mattanovich, 2013), a method that relies on serial cultivation under selective pressure, can facilitate the development of competitive strains. In *S. ovata* and *E. limosum*, ALE was for instance used to considerably accelerate growth on methanol in defined medium [from $0.010\ h^{-1}$ to $0.053\ h^{-1}$ in *S. ovata* (Tremblay et al., 2015), and from $0.022\ h^{-1}$ to $0.053\ h^{-1}$ in *E. limosum* (Pregnon et al.,

2022)]. Additionally, the tolerance towards methanol, which is highly cytotoxic, was considerably increased (yielding much higher growth rates and biomass yields at 2 % methanol than the wild type) (Kremp and Müller, 2021). In turn, these results underline the potential of ALE for increasing the tolerance of acetogenic hosts to methanol or formate, which might be a critical parameter for the industrial applicability of a bioproduction scenario based on methylotrophic/formatotrophic acetogens.

ALE can also be used following a targeted genome modification, in particular if that modification resulted in a low fitness. By serially sub-culturing cells growing under certain conditions, random mutations that increase the fitness of a mutant towards these conditions are selected over time. The double deletion mutant $\Delta hydBA/hydA2$ was for instance initially able to grow on 100 mM formate to an OD_{600} of 0.2, which, after 10 transfers, could be increased to an OD_{600} of 0.9 (Moon et al., 2024).

The development of appropriate metabolic engineering strategies is greatly facilitated by an accurate understanding of the physiology and metabolism of a microbe of interest. Systems-level analyses require a well-annotated, complete genome, which is available for the main methylotrophic and/or formatotrophic acetogens (Table 2). In turn, genomic data can be used directly to infer RNA and protein sequences and metabolic reactions, all of which are necessary to perform transcriptomic, proteomic and metabolomic analyses. In addition, the prediction of metabolic reactions enables the construction of genome-scale metabolic models (GSM), which connect all (known) metabolites and reactions in a cell in a computational representation of a metabolic network. The interest in GSMs stems from their potentially predictive value: modelling approaches based on GSMs and experimental data (e.g., -omics analyses) can, for example, effectively predict the outcome of genetic or environmental perturbations, which in turn has the potential to considerably accelerate rational metabolic and bioprocess engineering. The first GSM published for an acetogenic methylotroph/formatotroph was for *M. thermoacetica*, which gave critical insights into the bioenergetics of H_2/CO_2 conversion in this microbe (705 reactions and 698 metabolites) (Islam et al., 2015). Although a GSM has been constructed for *A. woodii*, it is currently not publicly available (Mesfin, 2020). Nevertheless, there is a metabolic core model available for *A. woodii* with 118 reactions effectively capturing central metabolism (Neuendorf et al., 2021). As a side note, computations in the following chapter were performed using this model. For *E. limosum*, the *A. woodii* model was modified to include the differences in co-factor specificity in the WLP.

GSMs can be coupled with physiological data, transcriptomics, proteomics and metabolomics to thoroughly characterize a particular methylotroph / formatotroph under a variety of experimental conditions. Linking substrate uptake to product formation data (i.e., titers, yields, rates, ideally obtained from continuous fermentations in chemostat mode) can notably be exploited to perform flux balance analysis (FBA), an approach that infers flux distribution within the cell based on observed substrate uptake and end metabolite/biomass produced at steady-state. FBA has been used to systematically investigate formatotrophic metabolism in *A. woodii*, allowing in particular the determination of ATP yield and maintenance costs, and predicting high energetic efficiency for formate-based bioproduction scenarios in *A. woodii* (Neuendorf et al., 2021).

Omics data can naturally provide highly valuable insights even without a GSM. In *E. limosum*, chemostat cultures grown on formate, methanol + CO_2 and methanol + formate were recently comprehensively analyzed using a combination of phenomics, proteomics and metabolomics approaches (Wood et al., 2023a; b). On formate, metabolomics revealed a complete redistribution of the carbon flux (compared to typical acetogens), with pyruvate accumulation and the production of a (probably novel) unknown metabolite (Wood et al., 2023a). Measurement of intracellular ATP, NAD and acetyl-CoA concentrations indicated an energy deficit, possibly related to formate-mediated stress. On methanol, metabolomics indicated that formate was better suited

than CO₂ as a co-substrate for alcohol production, as the NADH/NAD⁺ ratio was significantly higher in this case (a higher ratio is typically linked with solvent production in other acetogens) (Wood et al., 2021). Surprisingly, however, no alcohol was formed under any of the methylotrophic conditions tested. Thermodynamic and kinetic modelling of key enzymes within the central carbon metabolism indicated that a possible bottleneck preventing butanol production could be the aldehyde ferredoxin oxidoreductase (AOR). Indeed, AOR activity towards acid formation could outcompete ADH-driven alcohol formation, suggesting that the deletion of the *aor* gene could promote butanol formation in *E. limosum* under certain conditions.

When it comes to the characterization of acetogens, not all omics are born equal. Indeed, the regulation of acetogen metabolism is often considered to be primarily mediated at the post-translational level, that is, the directionality of fluxes is often largely independent of gene expression (Heffernan et al., 2022; Mahamkali et al., 2020). However, there are significant hurdles to performing metabolomics in acetogens that may prove difficult to overcome (Heffernan et al., 2022). First, the quantification of oxygen sensitive species, such as THF derivatives, is difficult and requires sampling under anaerobic conditions (De Souza Pinto Lemgruber et al., 2018). Quantification of the crucial oxidized/reduced ferredoxins poses additional challenges (notably related to large protein and cognate charge quantification in metabolomics workflows). Thermodynamic flux balance analysis (TFBA) has been used in "*C. autoethanogenum*" to estimate ferredoxin quantities (as well as the levels of intracellular exchange metabolites) (Mahamkali et al., 2020), and such an approach could potentially also be applied to methylotrophic and formatotrophic acetogens. Constraining ferredoxin concentration, however, provides only an indirect measure, which may negatively affect the accuracy of the model. As a result, future methodological improvements enabling system-scale quantification of ferredoxin concentration and charge would significantly advance the characterization of acetogen metabolism.

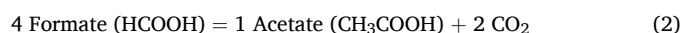
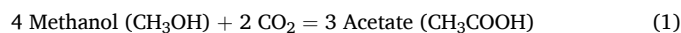
Metabolomics approaches using ¹³C-labelling may considerably improve metabolite quantitation and, in addition, identify how metabolites are connected in a metabolic network by accurately tracing the metabolic fate of labelled carbon within a given substrate (in an analysis referred to as metabolic flux analysis, MFA). For C1-utilizing microbes, this topic has been recently reviewed by Hoyt and Woolston (Hoyt and Woolston, 2022). In short, the main obstacle to using MFA for C1 microbes lies in the intrinsic nature of their substrate, as they present only a single carbon moiety, and MFA typically relies on the differential carbon fate of labelled isotopes placed at specific position on a substrate (e.g., [U-¹³C₆]-glucose). As a result, feeding a ¹³C-labelled C1 substrate will result in uniform labelling of the entire carbon pool of the cell, preventing analysis. Co-substrate feeding has been used to overcome this problem in the acetogen *Clostridium drakei* (using labelled fructose and bicarbonate) (Song et al., 2020), as in that case differential labelling is expected upon simultaneous uptake of both substrates. Alternatively, transient labelling can be used to perform MFA on C1 substrates. Transient labelling relies on replacing the labelled or unlabeled C1 source with either its labelled or unlabeled counterpart at a given time and measuring the ¹³C label distribution in the metabolic network over time. MFA based on transient labelling has been applied to the acetogens *Clostridium carboxidivorans* and *Clostridium ljungdahlii* to gain insight into their respective metabolisms. However, despite the progress made in these various acetogens, MFA has not yet been applied to acetogens grown under methylotrophic and formatotrophic conditions.

5. Bioenergetics of acetogenesis during methylotrophic and formatotrophic growth

To quantitatively assess the potential for acetogenic upgrading of the liquid C1 feedstocks methanol (+CO₂) and formate into value-added products, both the pathway stoichiometry and bioenergetics of the biocatalyst need to be considered. The latter is particularly crucial for

acetogens, as their lifestyle severely restricts ATP availability. Consequently, growth and metabolism are tightly coupled to the formation of the ATP-yielding product acetate. Whether a desired feedstock-to-product conversion with high yield (i.e., as the sole product) is feasible in an acetogenic biocatalyst is therefore dictated by the bioenergetics.

The stoichiometry informs about the carbon and energy efficiency of the conversion and is solely dependent on the properties of the feedstocks and products. Using the concepts of available electrons $\sum e^-$ and degree of reduction $\bar{\alpha}$ of both the substrates and products of the desired microbial conversion, the reaction stoichiometry can be assessed. The three feedstocks considered for the analysis of this study are methanol, formate, and CO₂, which have six, two, and zero available electrons. The degree of reduction can be obtained by dividing the number of available electrons by the number of carbon atoms of a compound. Since all three feedstocks are made up from a single carbon atom, the degree of reduction equals the number of available electrons. When considering acetate (CH₃COOH, $\sum e^- = 8$, $\bar{\alpha} = 4$) as the main metabolic end product of many acetogens, the pathway stoichiometry from different substrates can be explained. Reactions 1 and 2 show the reaction stoichiometry for acetogenesis from acetate and methanol (+CO₂) and formate:



To supply eight electrons to make one acetate, four formate are required, lowering the carbon efficiency of the conversion to 50 % due to the release of 2 CO₂, which comprises carbon without available electrons. In contrast, for conversion of the reduced feedstock methanol into the less reduced product acetate, additional CO₂ is required. Four mol methanol contain 24 available electrons, which matches the 24 available electrons in three mol acetate. However, to close the carbon balance, an additional two carbon atoms are needed without supplying extra electrons, which can be supplied as CO₂.

Fig. 2 shows the carbon efficiency for formate-to-product conversion as a function of the degree of reduction of the product. Using the highly reduced product hexanol (C₆H₁₄O, $\sum e^- = 36$, $\bar{\alpha} = 6$) as an additional example, carbon efficiency for formate decreases as the degree of reduction of the product increases. To supply the electrons for hexanol formation, 18 mol formate are required but only six are converted into hexanol and the rest is oxidized to CO₂, lowering the carbon efficiency to 33 %.

In case of the reduced feedstock methanol carbon efficiency is not dependent on the degree of reduction of the product. Rather, the ratio with which the more highly oxidized co-substrate CO₂ can be supplied is dictated by how reduced the target product is (Fig. 2b). If the degree of reduction of the product is lower than six ($\bar{\alpha}$ of methanol), additional carbon from CO₂ can be converted.

In case of co-supplying methanol and formate to the system, there is a similar correlation between the possible formate:methanol ratio and the degree of reduction of the product. However, in this case, additional electrons are also delivered by the co-substrate formate, resulting in a different ratio compared to CO₂:methanol (Fig. 2c). Moreover, the carbon efficiency is also affecting the formate:methanol ratio, where lower carbon efficiencies result in higher formate:methanol ratios and *vice versa*.

Using this framework, it is possible to design mixed feed systems meeting defined criteria in terms of product yields and carbon efficiency. Another important aspect for converting liquid C1 feedstocks is the energy efficiency of the system. Liquid C1 feedstocks would ideally be generated using renewable energy, a precious commodity that should be used as efficiently as possible, also considering the tight economics of the low value-high volume products considered in this analysis. The energy efficiency was analyzed using the combustion enthalpies $\Delta_c H^\circ$ liquid (in kJ mol⁻¹) of the feedstocks and products and the calculated theoretical yields (see Supplementary Material). For all products

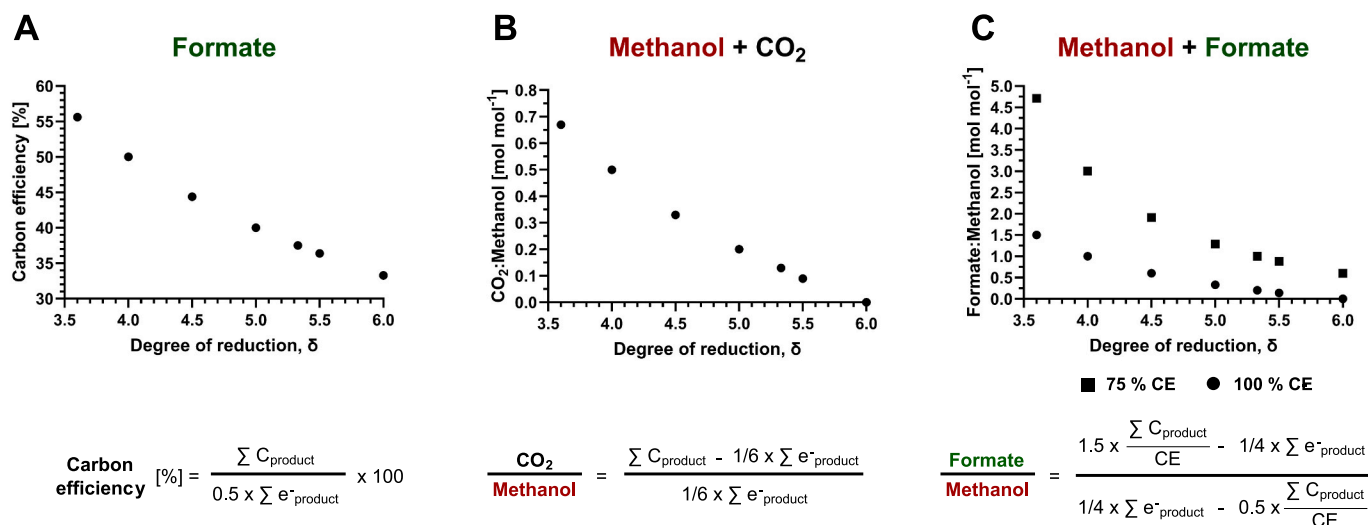


Fig. 2. Results of the stoichiometric analysis for the synthesis of the products shown in Fig. 1 from methanol (+CO₂), formate or methanol + formate. $\sum C_{\text{product}}$: number of carbon atoms in product, $\sum e^{-}_{\text{product}}$: number of electrons in the product, CE: carbon efficiency.

(Fig. 1) considered in the analysis shown in Fig. 2, the energy efficiency of the conversion was between 91 and 97 % (see Supplementary Material for details). However, the actual carbon and energy efficiency of a microbial production system is somewhat lower than the theoretical values due to the resource demand (carbon and energy) of the biocatalyst for growth and cell maintenance. In this context, acetogens with their efficient lifestyle again represent interesting bioproduction hosts as only a small amount of the carbon and energy of the feedstock (~5–7 %) is diverted from product formation for growth (Neuendorf et al., 2021; Novak et al., 2021; Valgepea et al., 2017).

As outlined above, the bioenergetics of the biocatalyst comprises the second dimension to be considered when designing a “liquid” C1 feedstock to product bioproduction scenario. As discussed in Section 2 Variants of the Wood-Ljungdahl pathway, acetogens metabolize C1 compounds through the WLP but the entry point of a C1 compound varies depending on its degree of reduction. As a result, reversible operation of both the methyl and carbonyl branch of the pathway is required to either reduce C1 compounds or to oxidize them partly to provide reduction equivalents for the rest of the substrate to undergo reduction (Fig. 1). Ultimately, the WLP yields acetyl-CoA by combining one C1 moiety each from the methyl and carbonyl branch, requiring six and two electrons for reduction respectively when CO₂ is the feedstock of the WLP. Despite the fixed stoichiometry of how individual C1 feedstocks are processed through the WLP, the bioenergetics might vary depending on the co-factors involved in the individual reduction and/or oxidation steps of the pathway. Since the ability of either the Rnf or Ech complex to generate a membrane gradient depends on the oxidation of Fd²⁻, there is a direct link between the pool of Fd²⁻ available during growth on a given C1 compound (and H₂ as electron donor) and the amount of ATP generated. Consequently, Fd²⁻ has been named an “ideal electron donor in an anoxic world” (Kuhns et al., 2020). The reason for this is the low redox potential of Fd/Fd²⁻ ($E_0' = -450$ – 500 mV) compared to the other electron carriers involved in acetogen metabolism: H₂/H⁺ ($E_0' = -414$ mV), NAD(P)H/NAD(P)⁺ ($E_0' = 320$ mV) (Schuchmann and Müller, 2014). Using this logic, ATP production of acetogens might be enhanced by increasing Fd²⁻ generation during substrate oxidation and by utilization of electron carriers with higher redox potentials such as NADH for reduction steps, e. g., in the WLP or product pathways.

To quantitatively assess the feasibility of substrate-product-biocatalyst combinations for upgrading formate and/or methanol (+CO₂) into value-added products, stoichiometric modeling was performed using the two model acetogens mainly employed for formate and

methanol utilization, *A. woodii* and *E. limosum*. Four substrate combinations (formate, formate + methanol with 100 % and 75 % carbon efficiency and methanol + CO₂), 17 different C2–C6 products formed via 21 metabolic pathways and two different metabolic backgrounds in *A. woodii* (HDCR or Fd-dependent formate dehydrogenase) and *E. limosum* (bifurcating and non-bifurcating MTHFR) were analyzed, resulting in a total of 336 ATP yields, expressed as mol ATP per mol of product (Fig. 3a-h). All scenarios were simulated using maximizing ATP production as the objective function, with growth set to zero and assuming formation of the respective product as sole metabolite (i.e., without acetate formation).

Using acetate as the base case, methanol results in higher ATP yields per mol acetate (*A. woodii*: +0.83 and *E. limosum*: +0.75) compared to formate (*A. woodii*: +0.30 and *E. limosum*: +0.25) in wild-type *A. woodii* and *E. limosum* (non-bifurcating MTHFR). This is further supported by the fact that only 38 % of all tested substrate-product scenarios are bioenergetically feasible when formate is the substrate in these strains (Table 3), whereas 62–81 % of all scenarios are feasible when methanol (+CO₂ or formate) is used. Among the feasible scenarios for formate are products which either involve synthesis of ATP as part of the pathway (e.g., acetate, butyrate) or where Fd²⁻ is generated and a significant amount of NADH is consumed in the product pathway (e.g., butyrate, butanol, hexanoate, hexanol). This is consistent with the above highlighted role of maximizing Fd²⁻ generation to drive a high flux through the Rnf complex, which boosts ATP synthesis if the generated NADH can be recycled (as is the case for the synthesis of reduced products).

For methanol, infeasible scenarios were typically found for products which require pyruvate instead of acetyl-CoA as the precursor (e.g., acetoin, 2,3-butanediol, butanone, 2-butanol, citramalate) or in case ATP is consumed in the product pathway (e.g., isobutene). The hypothesized electron-bifurcating mode of operation of the MTHFR in *E. limosum* generally lowers ATP yields when methanol serves as the substrate as Fd²⁻ is required during reverse operation of the methyl branch of the WLP. This lowers the total number of feasible scenarios. In contrast, if formate serves as the substrate under the same conditions, the total number of feasible scenarios is increased from 38 to 62 % as an additional Fd²⁻ is generated and an additional NADH consumed compared to the non-bifurcating scenario.

In the context of bioproduction, improving the bioenergetics is highly interesting as it could significantly expand the product spectrum from formate. One possible way to achieve this objective might be to create a direct link between formate oxidation and Fd²⁻ generation. Replacing the native H₂-dependent CO₂ reductase (HDCR) in *A. woodii*

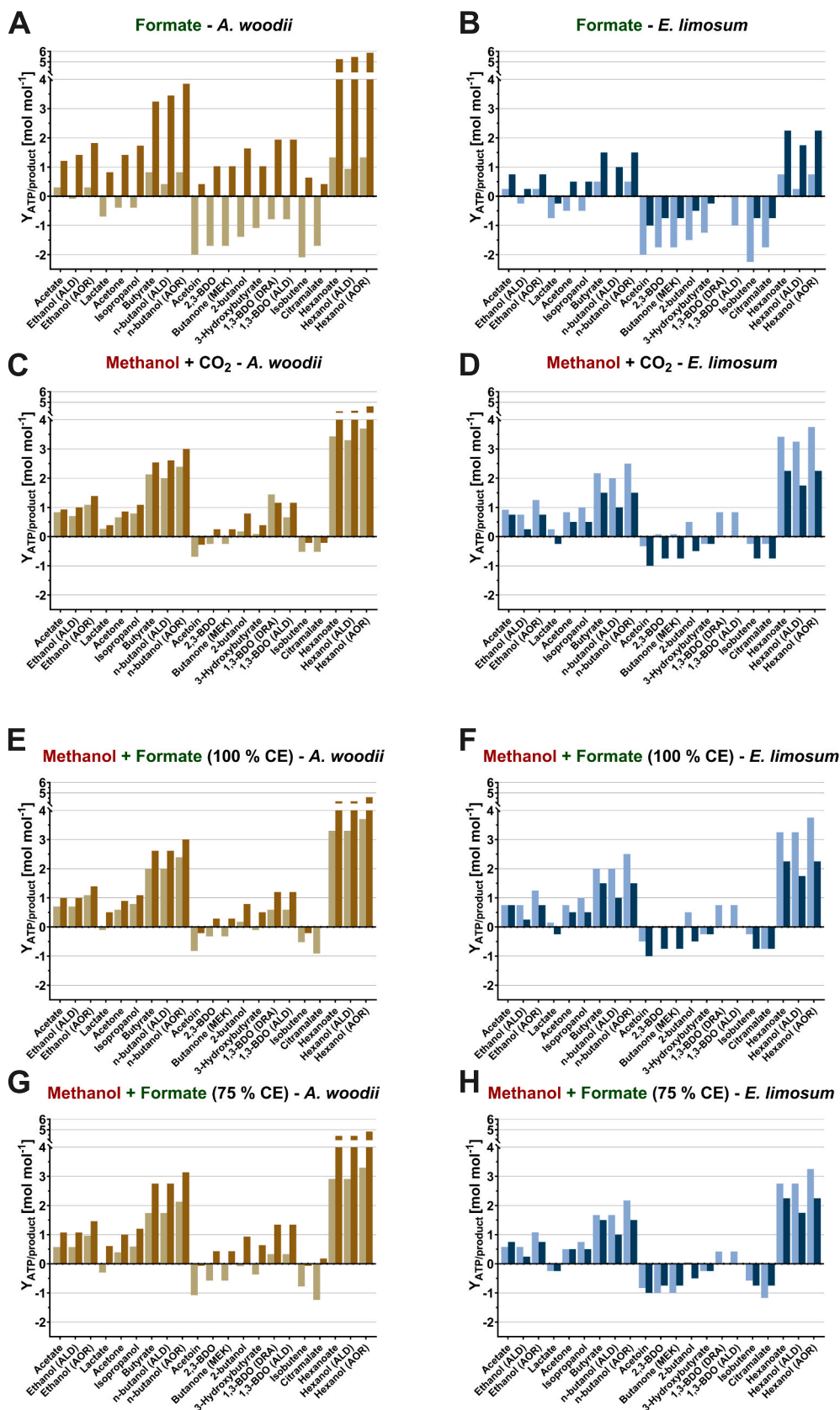


Fig. 3. Results of the bioenergetic analysis for the conversion of methanol and formate into 17 different C2 – C6 products in *A. woodii* and *E. limosum*. Two different metabolic backgrounds in *A. woodii* (light brown, wild type/HDCR or dark brown, Fd-dependent formate dehydrogenase) and *E. limosum* (light blue, bifurcating and dark blue, non-bifurcating methylene-THF reductase) were considered, each with formate (A, *A. woodii* and B, *E. limosum*), methanol + CO₂ (C, *A. woodii* and D, *E. limosum*) and methanol + formate (E and G, *A. woodii* and F and H, *E. limosum*) as substrates. All values shown are mol ATP formed per mol product. The

simulations were constrained to exclusively allow formation of the respective metabolic product (i. e., no acetate formation). CE: carbon efficiency. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Summary of the bioenergetics analysis of 336 substrate-product-biocatalyst combinations.

Feedstock	<i>A. woodii</i>		<i>E. limosum</i>	
	Wildtype (HDCR)	Evolved/Engineered (Fd-FDH)	Non-bifurcating MTHFR	Bifurcating MTHFR
Formate	38	100	38	62
Methanol + CO ₂	76	90	81	62
Methanol + Formate (100 %) ¹	67	86	71	62
Methanol + Formate (75 %) ²	62	90	67	62

Note: stoichiometry of methanol and formate was fixed to achieve 100 % (1) and 75 % (2) carbon efficiency.

Numbers shown are % feasible scenarios. **HDCR:** H₂-dependent CO₂ reductase, **Fd-FDH:** ferredoxin-dependent formate dehydrogenase, **MTHFR:** methylene-THF reductase. Other redox partners for *A. woodii* and *E. limosum* (see Fig. 1). The complete results are displayed in Fig. 3a-h and listed in Table S2.

with a ferredoxin-dependent formate dehydrogenase (Fd-FDH) for example dramatically increases ATP production 4-fold from 0.30 to 1.21 ATP per mol acetate. Such a strain of *A. woodii* does not natively exist but the purified HDCR has been shown to accept Fd as an electron acceptor in addition to H⁺. Indeed, an engineered and evolved strain of *A. woodii* lacking the hydrogenase subunit of the HDCR has recently been shown to grow on formate with an improved biomass yield and higher intracellular ATP concentrations (Moon et al., 2024). A SNP analysis of the engineered and adapted strain revealed a mutation in the iron-sulfur containing protein HycB2, which is part of the HDCR complex and is involved in electron transport. Indeed, Fd-dependent CO₂ hydrogenation (25-fold) and formate oxidation (6.7-fold) activity was significantly enhanced in the engineered and adapted strain compared to the engineered but non-adapted strain.

Besides improving native Fd-dependent formate oxidation activity, rational engineering by expression of a strictly Fd-dependent formate dehydrogenase (e.g., from *Clostridium pasteurianum*, Jungermann et al., 1970) might be an alternative way of implementing such a metabolic module in acetogenic hosts. In the case of *A. woodii*, the implementation of a Fd-dependent FDH system dramatically increases ATP yields with all 21 product pathways becoming feasible for formate as the substrate, compared to only 38 % for the wild type. Additionally, between 86 and 90 % of all scenarios are feasible when methanol (+CO₂ or formate) serves as the feedstock in a strain modified in this way. Despite the obvious advantage for the bioenergetics, the adapted strain showed lower formate consumption rates in serum bottle experiments compared to the wild type (Moon et al., 2024). This can be explained by the fact that the thermodynamics of the formate oxidation are altered, from $\Delta G_0' = -4 \text{ kJ mol}^{-1}$ when H⁺ is the electron acceptor to $\Delta G_0' = +4 \text{ kJ mol}^{-1}$ when Fd serves as redox partner. Consequently, future research in this direction should be guided by careful selection of the cultivation conditions (high formate, low CO₂/bicarbonate concentrations, Moon et al., 2024) as well as further strain adaptation to enable adjustment of the thermodynamic landscape of the metabolic network to the altered conditions to improve pathway functionality. As outlined in section 2 Variants of the Wood-Ljungdahl pathway, altering the pyruvate-forming reaction from PFOR to PFL might be beneficial due to lower consumption of Fd²⁻. Indeed, model simulations show that in an *A. woodii* strain, using PFL instead of PFOR makes lactate formation from formate bioenergetically feasible (+0.21 ATP per mol of lactate).

Strategies other than altering the co-factor specificity of key enzymes of the WLP to improve the bioenergetics have been reviewed by Katsy and Müller (2020) for autotrophic bioproduction with acetogens, which might also be applicable for methanol- and formate-based bioproduction. Collectively, this stoichiometric and bioenergetic analysis shows the potential of using the liquid C1 feedstocks methanol and formate to generate chemicals and fuels of interest and outlines possibilities to increase the number of feasible scenarios.

6. Conclusion

The high carbon and energy efficiency combined with the tightly growth-coupled product formation renders acetogens attractive platform hosts for truly continuous bioproduction scenarios to upgrade methanol and formate into value-added products. Outstanding scientific and technical questions remain, including the need for a deeper understanding of methanol and formate metabolism in acetogens, which can be facilitated by quantitative studies in defined bioreactor cultivations. Additionally, bioprocessing across different scales (from lab to pilot scale) is needed to support process optimization and strain development. Furthermore, the systematic application of recently developed genome editing tools is essential for creating novel strains, such as those with new product pathways, improved bioenergetics, or enhanced methanol utilization capabilities. Additionally, synthetic co-cultures could be used for methanol and formate upgrading by combining metabolic features for a desired conversion scenario.

Advancing bioprocessing strategies could enable the industrial use (typically requiring product titers, rates and yields of 50 g L⁻¹, 3 g L⁻¹h⁻¹ and 80 %, (Van Dien, 2013) of methanol and formate as renewable feedstocks and acetogens as biocatalysts, placing them at the heart of a circular and sustainable bioeconomy.

CRedit authorship contribution statement

Eugenio Pettinato: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Rémi Hocq:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Stefan Pflügl:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Funding acquisition, Conceptualization.

Funding

Open access funding provided by TU Wien (TUW). This research was funded in whole or in part by the Austrian Science Fund (FWF) [<https://doi.org/10.55776/Y1513>]. For open access purposes, the author has applied a CC BY public copyright license to any author accepted manuscript version arising from this submission.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2025.132643>.

Data availability

Data will be made available on request.

References

- Alsarhan, L.M., Alayyar, A.S., Alqahtani, N.B., Khadry, N.H., 2021. Circular carbon economy (CCE): a way to invest CO₂ and protect the environment, a review. *Sustainability* 13, 11625. <https://doi.org/10.3390/su132111625>.
- Andresen, J.R., Ljungdahl, L.G., 1974. Nicotinamide adenine dinucleotide phosphate-dependent formate dehydrogenase from *Clostridium thermoaceticum*: purification and properties. *J. Bacteriol.* 120, 6–14. <https://doi.org/10.1128/jb.120.1.6-14.1974>.
- Arslan, K., Schoch, T., Höfele, F., Herrschaft, S., Oberlies, C., Bengelsdorf, F., Veiga, M.C., Dürre, P., Kennes, C., 2022. Engineering *Acetobacterium woodii* for the production of isopropanol and acetone from carbon dioxide and hydrogen. *Biotechnol. J.*, 2100515 <https://doi.org/10.1002/biot.202100515>.
- Bache, R., Pfennig, N., 1981. Selective isolation of *Acetobacterium woodii* on methoxylated aromatic acids and determination of growth yields. *Arch. Microbiol.* 130, 255–261. <https://doi.org/10.1007/BF00459530>.
- Bachleitner, S., Ata, Ö., Mattanovich, D., 2023. The potential of CO₂-based production cycles in biotechnology to fight the climate crisis. *Nat. Commun.* 14, 6978. <https://doi.org/10.1038/s41467-023-42790-6>.
- Baker, J.P., Sáez-Sáez, J., Jensen, S.I., Nielsen, A.T., Minton, N.P., 2022. A clean in-frame knockout system for gene deletion in *Acetobacterium woodii*. *J. Biotechnol.* 353, 9–18. <https://doi.org/10.1016/j.jbiotec.2022.05.013>.
- Balch, W.E., Schoberth, S., Tanner, R.S., Wolfe, R.S., 1977. *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. *Int. J. Syst. Bacteriol.* 27, 355–361. <https://doi.org/10.1099/00207713-27-4-355>.
- Basen, M., Geiger, I., Henke, L., Müller, V., 2018. A genetic system for the thermophilic acetogenic bacterium thermoanaerobacter kivui. *Appl. Environ. Microbiol.* 84, e02210–e02217. <https://doi.org/10.1128/AEM.02210-17>.
- Beck, M.H., Flaiz, M., Bengelsdorf, F.R., Dürre, P., 2020. Induced heterologous expression of the arginine deiminase pathway promotes growth advantages in the strict anaerobe *Acetobacterium woodii*. *Appl. Microbiol. Biotechnol.* 104, 687–699. <https://doi.org/10.1007/s00253-019-10248-9>.
- Bengelsdorf, F.R., Poehlein, A., Schiel-Bengelsdorf, B., Daniel, R., Dürre, P., 2016. Genome sequence of the acetogenic bacterium *Butyribacterium methylotrophicum* DSM 3468T. *Genome Announc.* 4, e01338. <https://doi.org/10.1128/genomeA.01338-16>.
- Bertsch, J., Müller, V., 2015. Bioenergetic constraints for conversion of syngas to biofuels in acetogenic bacteria. *Biotechnol. Biofuels* 8, 210. <https://doi.org/10.1186/s13068-015-0393-x>.
- Bertsch, J., Öppinger, C., Hess, V., Langer, J.D., Müller, V., 2015. Heterotrimeric NADH-oxidizing methylenetetrahydrofolate reductase from the acetogenic bacterium *Acetobacterium woodii*. *J. Bacteriol.* 197, 1681–1689. <https://doi.org/10.1128/JB.00048-15>.
- Bourgade, B., Millard, J., Humphreys, C.M., Minton, N.P., Islam, M.A., 2022. Enabling ethanologensis in *Moorella thermoacetica* through construction of a replicating shuttle vector. *Fermentation* 8, 585. <https://doi.org/10.3390/fermentation8110585>.
- Burger, Y., Schwarz, F.M., Müller, V., 2022. Formate-driven H₂ production by whole cells of *Thermoanaerobacter kivui*. *Biotechnol. Biofuels* 15, 48. <https://doi.org/10.1186/s13068-022-02147-5>.
- Buschhorn, H., Dürre, P., Gottschalk, G., 1989. Production and utilization of ethanol by the Homoacetogen *Acetobacterium woodii*. *Appl. Environ. Microbiol.* 55, 1835–1840. <https://doi.org/10.1128/AEM.55.7.1835-1840.1989>.
- Chang, et al., 1999. Formulation of defined media for carbon monoxide fermentation by *Eubacterium limosum* KIST612 and the growth characteristics of the bacterium. *J. Biosci. Bioeng.* 88, 682–685.
- Cheah, W.Y., Ling, T.C., Juan, J.C., Lee, D.-J., Chang, J.-S., Show, P.L., 2016. Biorefineries of carbon dioxide: From carbon capture and storage (CCS) to bioenergies production. *Bioresour. Technol.* 215, 346–356. <https://doi.org/10.1016/j.biortech.2016.04.019>.
- Chowdhury, N.P., Litty, D., Müller, V., 2022. Biosynthesis of butyrate from methanol and carbon monoxide by recombinant *Acetobacterium woodii*. *Int. Microbiol.* <https://doi.org/10.1007/s10123-022-00234-z>.
- Cotton, C.A., Claessens, N.J., Benito-Vaquero, S., Bar-Even, A., 2020. Renewable methanol and formate as microbial feedstocks. *Curr. Opin. Biotechnol.* 62, 168–180. <https://doi.org/10.1016/j.copbio.2019.10.002>.
- Daniel, S.L., Hsu, T., Dean, S.I., Drake, H.L., 1990. Characterization of the H₂- and CO₂-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. *J. Bacteriol.* 172, 4464–4471. <https://doi.org/10.1128/jb.172.8.4464-4471.1990>.
- Das, A., Fu, Z., Tempel, W., Liu, Z., Chang, J., Chen, L., Lee, D., Zhou, W., Xu, H., Shaw, N., Rose, J.P., Ljungdahl, L.G., Wang, B., 2007. Characterization of a corrinoid protein involved in the C1 metabolism of strict anaerobic bacterium *Moorella thermoacetica*. *Proteins* 67, 167–176. <https://doi.org/10.1002/prot.21094>.
- Dawson, L.F., Valiente, E., Faulds-Pain, A., Donahue, E.H., Wren, B.W., 2012. Characterisation of clostridium difficile biofilm formation, a role for Spo0A. *PLoS One* 7, e50527. <https://doi.org/10.1371/journal.pone.0050527>.
- De Souza Pinto Lemgruber, R., Valgepea, K., Hodson, M.P., Tappel, R., Simpson, S.D., Köpke, M., Nielsen, L.K., Marcellin, E., 2018. Quantitative analysis of tetrahydrofolate metabolites from clostridium autoethanogenesis. *Metabolomics* 14, 35. <https://doi.org/10.1007/s11306-018-1331-2>.
- Dietrich, H.M., Kremp, F., Öppinger, C., Ribaric, L., Müller, V., 2021. Biochemistry of methanol-dependent acetogenesis in *Eubacterium callanderi* KIST612. *Environ. Microbiol.* 1462–2920, 15643. <https://doi.org/10.1111/1462-2920.15643>.
- Dietrich, H.M., Müller, V., 2023. Ferredoxin as a physiological electron donor for carbon dioxide fixation to formate in a bacterial carbon dioxide reductase. *ACS Catal.* 13, 12374–12382. <https://doi.org/10.1021/acscatal.3c02753>.
- Dietrich, H.M., Righetto, R.D., Kumar, A., Wietrzynski, W., Trischler, R., Schuller, S.K., Wagner, J., Schwarz, F.M., Engel, B.D., Müller, V., Schuller, J.M., 2022. Membrane-anchored HDRC nanowires drive hydrogen-powered CO₂ fixation. *Nature* 607, 823–830. <https://doi.org/10.1038/s41586-022-04971-z>.
- Dijkhuizen, L., Hansen, T.A., Harder, W., 1985. Methanol, a potential feedstock for biotechnological processes. *Trends Biotechnol.* 3, 262–267. [https://doi.org/10.1016/0167-7799\(85\)90026-5](https://doi.org/10.1016/0167-7799(85)90026-5).
- Dragosits, M., Mattanovich, D., 2013. Adaptive laboratory evolution – principles and applications for biotechnology. *Microb. Cell Fact* 12, 64. <https://doi.org/10.1186/1475-2859-12-64>.
- Drake, H.L., Daniel, S.L., 2004. Physiology of the thermophilic acetogen *Moorella thermoacetica*. *Res. Microbiol.* 155, 422–436. <https://doi.org/10.1016/j.resmic.2004.03.003>.
- Dronsella, B., Orsi, E., Schulz-Mirbach, H., Benito-Vaquero, S., Yilmaz, S., Glatter, T., Bar-Even, A., Erb, T.J., Claessens, N.J., 2025. One-carbon fixation via the synthetic reductive glycine pathway exceeds yield of the Calvin cycle. *Nat. Microbiol.* 10, 646–653. <https://doi.org/10.1038/s41564-025-01941-9>.
- Dürre, P., Eikmanns, B.J., 2015. C1-carbon sources for chemical and fuel production by microbial gas fermentation. *Curr. Opin. Biotechnol.* 35, 63–72. <https://doi.org/10.1016/j.copbio.2015.03.008>.
- Ewis, D., Arslan, M., Khaled, M., Pant, D., Ba-Abbad, M.M., Amhamed, A., El-Naas, M. H., 2023. Electrochemical reduction of CO₂ into formate/formic acid: a review of cell design and operation. *Sep. Purif. Technol.* 316, 123811. <https://doi.org/10.1016/j.seppur.2023.123811>.
- Flaiz, M., Baur, T., Gaibler, J., Kröly, C., Dürre, P., 2022. Establishment of green- and red-fluorescent reporter proteins based on the fluorescence-activating and absorption-shifting tag for use in acetogenic and solventogenic anaerobes. *ACS Synth. Biol.* <https://doi.org/10.1021/acssynbio.1c00554>.
- Flaiz, M., Ludwig, G., Bengelsdorf, F.R., Dürre, P., 2021. Production of the biocommodities butanol and acetone from methanol with fluorescent FAST-tagged proteins using metabolically engineered strains of *Eubacterium limosum*. *Biotechnol. Biofuels* 14, 117. <https://doi.org/10.1186/s13068-021-01966-2>.
- Flaiz, M., Poehlein, A., Wilhelm, W., Mook, A., Daniel, R., Dürre, P., Bengelsdorf, F.R., 2024. Refining and illuminating acetogenic *Eubacterium* strains for reclassification and metabolic engineering. *Microb. Cell Fact.* 23, 24. <https://doi.org/10.1186/s12934-024-02301-8>.
- Fontaine, F.E., Peterson, W.H., McCoy, E., Johnson, M.J., Ritter, G.J., 1942. A new type of glucose fermentation by *Clostridium thermoaceticum*. *J. Bacteriol.* 43, 701–715. <https://doi.org/10.1128/jb.43.6.701-715.1942>.
- Heffernan, J.K., Mahamkali, V., Valgepea, K., Marcellin, E., Nielsen, L.K., 2022. Analytical tools for unravelling the metabolism of gas-fermenting Clostridia. *Curr. Opin. Biotechnol.* 75, 102700. <https://doi.org/10.1016/j.copbio.2022.102700>.
- Heffernan, J.K., Valgepea, K., de Souza Pinto Lemgruber, R., Casini, I., Plan, M., Tappel, R., Simpson, S.D., Köpke, M., Nielsen, L.K., Marcellin, E., 2020. Enhancing CO₂-valorization using clostridium autoethanogenesis for sustainable fuel and chemicals production. *Front. Bioeng. Biotechnol.* 8, 204. <https://doi.org/10.3389/fbioe.2020.0204>.
- Herzog, J., Mook, A., Guhl, L., Bäumler, M., Beck, M.H., Weuster-Botz, D., Bengelsdorf, F. R., Zeng, A., 2022. Novel synthetic co-culture of *Acetobacterium woodii* and *Clostridium drakei* using CO₂ and in situ generated H₂ for the production of caproic acid via lactic acid. *elc.202100169 Eng. Life Sci.* <https://doi.org/10.1002/elsc.202100169>.
- Hocq, R., Bottone, S., Gautier, A., Pflügl, S., 2023. A fluorescent reporter system for anaerobic thermophiles. *Front. Bioeng. Biotechnol.* 11, 1226889. <https://doi.org/10.3389/fbioe.2023.1226889>.
- Höfele, F., Schoch, T., Oberlies, C., Dürre, P., 2023. Heterologous production of isopropanol using metabolically engineered *Acetobacterium woodii* strains. *Bioengineering* 10, 1381. <https://doi.org/10.3390/bioengineering10121381>.
- Hoffmeister, S., Gerdom, M., Bengelsdorf, F.R., Linder, S., Flüchter, S., Öztürk, H., Blimke, W., May, A., Fischer, R.-J., Bahl, H., Dürre, P., 2016. Acetone production with metabolically engineered strains of *Acetobacterium woodii*. *Metab. Eng.* 36, 37–47. <https://doi.org/10.1016/j.ymben.2016.03.001>.
- Hoyt, K.O., Woolston, B.M., 2022. Adapting isotopic tracer and metabolic flux analysis approaches to study C1 metabolism. *Curr. Opin. Biotechnol.* 75, 102695. <https://doi.org/10.1016/j.copbio.2022.102695>.
- Hu, J., Zeng, C., Liu, G., Luo, H., 2024. Nitrogen-doped carbon dots boost microbial electrosynthesis via efficient extracellular electron uptake of acetogens. *Bioresour. Technol.* 412, 131390. <https://doi.org/10.1016/j.biortech.2024.131390>.
- Humphreys, J.R., Hebdson, S.D., Rohrer, H., Magnusson, L., Urban, C., Chen, Y.-P., Lo, J., 2022. Establishing *Butyribacterium methylotrophicum* as a platform organism for the production of biocommodities from liquid C1 metabolites. *Appl. Environ. Microbiol.* <https://doi.org/10.1128/aem.02393-21>.
- International Energy Agency, 2023. CO₂ Emissions in 2022. OECD. <https://doi.org/10.1787/12ad1e1a-en>.
- Islam, M.A., Zengler, K., Edwards, E.A., Mahadevan, R., Stephanopoulos, G., 2015. Investigating *Moorella thermoacetica* metabolism with a genome-scale constraint-based metabolic model. *Integr. Biol.* 7, 869–882. <https://doi.org/10.1039/C5IB00095E>.
- Iwasaki, Y., Kita, A., Yoshida, K., Tajima, T., Yano, S., Shou, T., Saito, M., Kato, J., Murakami, K., Nakashimada, Y., 2017. Homolactic acid fermentation by the

- genetically engineered thermophilic homoacetogen *Moorella thermoacetica* ATCC 39073. *Appl. Environ. Microbiol.* 83. <https://doi.org/10.1128/AEM.00247-17>.
- Jeong, J., Kim, J.-Y., Park, B., Choi, I.-G., Chang, I.S., 2020. Genetic engineering system for syngas-utilizing acetogen, *Eubacterium limosum* KIST612. *Bioresour. Technol. Rep.* 11, 100452. <https://doi.org/10.1016/j.biteb.2020.100452>.
- Jia, D., Deng, W., Hu, P., Jiang, W., Gu, Y., 2023. Thermophilic *Moorella thermoacetica* as a platform microorganism for C1 gas utilization: physiology, engineering, and applications. *Bioresour. Bioprocess* 10, 61. <https://doi.org/10.1186/s40643-023-00682-z>.
- Jin, S., Kang, S., Bae, J., Lee, H., Cho, B.-K., 2022. Development of CO gas conversion system using high CO tolerance biocatalyst. *Chem. Eng. J.* 449, 137678. <https://doi.org/10.1016/j.cej.2022.137678>.
- Jungermann, K., Kirchner, H., Thauer, R.K., 1970. Ferredoxin dependent CO₂ reduction to formate in *Clostridium pasteurianum*. *Biochem. Biophys. Res. Commun.* 41, 682–689. [https://doi.org/10.1016/0006-291X\(70\)90067-7](https://doi.org/10.1016/0006-291X(70)90067-7).
- Kang, S., Boshell, F., Goepfert, A., Prakash, S.G., Landäl, I., Saygin, D., 2021. Innovation outlook: renewable methanol. International Renewable Energy Agency, Abu Dhabi.
- Kang, S., Song, Y., Jin, S., Shin, J., Bae, J., Kim, D.R., Lee, J.-K., Kim, S.C., Cho, S., Cho, B.-K., 2020. Adaptive laboratory evolution of *Eubacterium limosum* ATCC 8486 on carbon monoxide. *Front. Microbiol.* 11, 402. <https://doi.org/10.3389/fmicb.2020.00402>.
- Kato, J., Fujii, T., Kato, S., Wada, K., Watanabe, M., Nakamichi, Y., Aoi, Y., Morita, T., Murakami, K., Nakashimada, Y., 2024a. Genetic engineering of a thermophilic acetogen, *Moorella thermoacetica* Y72, to enable acetoin production. *Front. Bioeng. Biotechnol.* 12, 1398467. <https://doi.org/10.3389/fbioe.2024.1398467>.
- Kato, J., Matsuo, T., Takemura, K., Kato, S., Fujii, T., Wada, K., Nakamichi, Y., Watanabe, M., Aoi, Y., Morita, T., Murakami, K., Nakashimada, Y., 2024b. Isopropanol production via the thermophilic bioconversion of sugars and syngas using metabolically engineered *Moorella thermoacetica*. *Biotechnol. Biofuels* 17, 13. <https://doi.org/10.1186/s13068-024-02460-1>.
- Kato, J., Takemura, K., Kato, S., Fujii, T., Wada, K., Iwasaki, Y., Aoi, Y., Matsushika, A., Murakami, K., Nakashimada, Y., 2021. Metabolic engineering of *Moorella thermoacetica* for thermophilic bioconversion of gaseous substrates to a volatile chemical. *AMB Expr.* 11, 59. <https://doi.org/10.1186/s13568-021-01220-w>.
- Katsyav, A., Müller, V., 2020. Overcoming energetic barriers in acetogenic C1 conversion. *Front. Bioeng. Biotechnol.* 8, 621166. <https://doi.org/10.3389/fbioe.2020.621166>.
- Katsyav, A., Jain, S., Basen, M., Müller, V., 2021. Electron carriers involved in autotrophic and heterotrophic acetogenesis in the thermophilic bacterium *Thermoanaerobacter kivui*. *Extremophiles*. <https://doi.org/10.1007/s00792-021-01247-8>.
- Kerby, R., Zeikus, J.G., 1987. Anaerobic catabolism of formate to acetate and CO₂ by *Butyrivibrio methylotrophicus*. *J. Bacteriol.* 169, 2063–2068. <https://doi.org/10.1128/jb.169.5.2063-2068.1987>.
- Kim, J.-Y., Park, S., Jeong, J., Lee, M., Kang, B., Jang, S.H., Jeon, J., Jang, N., Oh, S., Park, Z.-Y., Chang, I.S., 2021. Methanol supply speeds up synthesis gas fermentation by methylotrophic-acetogenic bacterium, *Eubacterium limosum* KIST612. *Bioresour. Technol.* 321, 124521. <https://doi.org/10.1016/j.biortech.2020.124521>.
- Kim, S., Lindner, S.N., Aslan, S., Yishai, O., Wenk, S., Schann, K., Bar-Even, A., 2020. Growth of *E. coli* on formate and methanol via the reductive glycine pathway. *Nat. Chem. Biol.* 16, 538–545. <https://doi.org/10.1038/s41589-020-0473-5>.
- Kita, A., Iwasaki, Y., Yano, S., Nakashimada, Y., Hoshino, T., Murakami, K., 2013. Isolation of thermophilic acetogens and transformation of them with the *pyrF* and *kan^r* Genes. *Biosci. Biotech. Bioch.* 77, 301–306. <https://doi.org/10.1271/bbb.120720>.
- Köpke, M., Simpson, S.D., 2020. Pollution to products: recycling of ‘above ground’ carbon by gas fermentation. *Curr. Opin. Biotechnol.* 65, 180–189. <https://doi.org/10.1016/j.copbio.2020.02.017>.
- Kremp, F., Müller, V., 2021. Methanol and methyl group conversion in acetogenic bacteria: biochemistry, physiology and application. *FEMS Microbiol. Rev.* 45, fuaa040. <https://doi.org/10.1093/femsre/fuaa040>.
- Kremp, F., Poehlein, A., Daniel, R., Müller, V., 2018. Methanol metabolism in the acetogenic bacterium *Acetobacterium woodii*. *Environ. Microbiol.* 20, 4369–4384. <https://doi.org/10.1111/1462-2920.14356>.
- Kremp, F., Roth, J., Müller, V., 2022. A third way of energy conservation in acetogenic bacteria. *Microbiol. Spectr.* 10, e01385-22. <https://doi.org/10.1128/spectrum.01385-22>.
- Kuhns, M., Schuchmann, V., Schmidt, S., Friedrich, T., Wiechmann, A., Müller, V., 2020. The Rnf complex from the acetogenic bacterium *Acetobacterium woodii*: purification and characterization of RnfC and RnfB. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 148263. <https://doi.org/10.1016/j.bbabi.2020.148263>.
- Lee, J.A., Kim, H.U., Na, J.-G., Ko, Y.-S., Cho, J.S., Lee, S.Y., 2023. Factors affecting the competitiveness of bacterial fermentation. *Trends Biotechnol.* 41, 798–816. <https://doi.org/10.1016/j.tibtech.2022.10.005>.
- Leigh, J.A., Mayer, F., Wolfe, R.S., 1981. *Acetogenium kivui*, a new thermophilic hydrogen-oxidizing acetogenic bacterium. *Arch. Microbiol.* 129, 275–280. <https://doi.org/10.1007/BF00414697>.
- Levi, P.G., Cullen, J.M., 2018. Mapping global flows of chemicals: from fossil fuel feedstocks to chemical products. *Environ. Sci. Technol.* 52, 1725–1734. <https://doi.org/10.1021/acs.est.7b04573>.
- Li, P., Gong, S., Li, C., Liu, Z., 2022. Analysis of routes for electrochemical conversion of CO₂ to methanol. *Clean Energy* 6, 202–210. <https://doi.org/10.1093/ce/zkac007>.
- Liew, F., Martin, M.E., Tappel, R.C., Heijstra, B.D., Mihalcea, C., Köpke, M., 2016. Gas fermentation—A flexible platform for commercial scale production of low-carbon-fuels and chemicals from waste and renewable feedstocks. *Front. Microbiol.* 7. <https://doi.org/10.3389/fmicb.2016.00694>.
- Litty, D., Kremp, F., Müller, V., 2022. One substrate, many fates: different ways of methanol utilization in the acetogen *Acetobacterium woodii*. *Environ. Microbiol.* 1462-2920, 16011. <https://doi.org/10.1111/1462-2920.16011>.
- Litty, D., Müller, V., 2021. Butyrate production in the acetogen *Eubacterium limosum* is dependent on the carbon and energy source. *Microb. Biotechnol.* 1751-7915, 13779. <https://doi.org/10.1111/1751-7915.13779>.
- Liu, Z., Wang, K., Chen, Y., Tan, T., Nielsen, J., 2020. Third-generation biorefineries as the means to produce fuels and chemicals from CO₂. *Nat. Catal.* 3, 274–288. <https://doi.org/10.1038/s41929-019-0421-5>.
- Ljungdhal, L.G., 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. *Annu. Rev. Microbiol.* 40, 415–450. <https://doi.org/10.1146/annurev.mi.40.100186.002215>.
- Madjarof, J., Soares, R., Paquete, C.M., Louro, R.O., 2022. *Sporomusa ovata* as catalyst for bioelectrochemical carbon dioxide reduction: a review across disciplines from microbiology to process engineering. *Front. Microbiol.* 13, 913311. <https://doi.org/10.3389/fmicb.2022.913311>.
- Mahamkali, V., Valgepea, K., De Souza Pinto Lemgruber, R., Plan, M., Tappel, R., Köpke, M., Simpson, S.D., Nielsen, L.K., Marcellin, E., 2020. Redox controls metabolic robustness in the gas-fermenting acetogen *Clostridium autoethanogenum*. *Proc. Natl. Acad. Sci. USA* 117, 13168–13175. <https://doi.org/10.1073/pnas.1919531117>.
- Mauerhofer, A.M., Müller, S., Bartik, A., Benedikt, F., Fuchs, J., Hammerschmid, M., Hofbauer, H., 2021. Conversion of CO₂ during the DFB biomass gasification process. *Biomass Conv. Bioref.* 11, 15–27. <https://doi.org/10.1007/s13399-020-00822-x>.
- Millard, J., Agius, A., Zhang, Y., Soucaille, P., Minton, N., 2023. Exploitation of a type 1 toxin-antitoxin system as an inducible counter-selective marker for genome editing in the acetogen *Eubacterium limosum*. *Microorganisms* 11, 1256. <https://doi.org/10.3390/microorganisms11051256>.
- Möller, B., Öbmer, R., Howard, B.H., Gottschalk, G., Hippe, H., 1984. *Sporomusa*, a new genus of gram-negative anaerobic bacteria including *Sporomusa sphaeroides* spec. nov. and *Sporomusa ovata* spec. nov. *Arch. Microbiol.* 139, 388–396. <https://doi.org/10.1007/BF00408385>.
- Mook, A., Beck, M.H., Baker, J.P., Minton, N.P., Dürre, P., Bengelsdorf, F.R., 2022. Autotrophic lactate production from H₂ + CO₂ using recombinant and fluorescent FAST-tagged *Acetobacterium woodii* strains. *Appl. Microbiol. Biotechnol.* 106, 1447–1458. <https://doi.org/10.1007/s00253-022-11770-z>.
- Moon, J., Dönig, J., Kramer, S., Poehlein, A., Daniel, R., Müller, V., 2021. Formate metabolism in the acetogenic bacterium *Acetobacterium woodii*. *Environ. Microbiol.* <https://doi.org/10.1111/1462-2920.15598>.
- Moon, J., Müller, V., 2021. Physiology and genetics of ethanogenesis in the acetogenic bacterium *Acetobacterium woodii*. *Environ. Microbiol.* <https://doi.org/10.1111/1462-2920.15739>.
- Moon, J., Poehlein, A., Daniel, R., Müller, V., 2024. Redirecting electron flow in *Acetobacterium woodii* enables growth on CO and improves growth on formate. *Nat. Commun.* 15, 5424. <https://doi.org/10.1038/s41467-024-49680-5>.
- Moon, J., Schubert, A., Waschinger, L.M., Müller, V., 2023. Reprogramming the metabolism of an acetogenic bacterium to homoformatogenesis. *ISME J.* 1–9. <https://doi.org/10.1038/s41396-023-01411-2>.
- Moreira, J.P.C., Heap, J.T., Alves, J.L., Domingues, L., 2023. Developing a genetic engineering method for *Acetobacterium wieringae* to expand one-carbon valorization pathways. *Biotechnol. Biofuels Bioproducts* 16, 24. <https://doi.org/10.1186/s13068-023-02259-6>.
- Nattermann, M., Wenk, S., Pfister, P., He, H., Lee, S.H., Szymanski, W., Guntermann, N., Zhu, F., Nickel, L., Wallner, C., Zarzycki, J., Paczia, N., Gaißert, N., Franció, G., Leitner, W., Gonzalez, R., Erb, T.J., 2023. Engineering a new-to-nature cascade for phosphate-dependent formate to formaldehyde conversion in vitro and in vivo. *Nat. Commun.* 14, 2682. <https://doi.org/10.1038/s41467-023-38072-w>.
- Neuendorf, C.S., Vignolle, G.A., Derntl, C., Tomin, T., Novak, K., Mach, R.L., Birner-Grünberger, R., Pflügl, S., 2021. A quantitative metabolic analysis reveals *Acetobacterium woodii* as a flexible and robust host for formate-based bioproduction. *Metab. Eng.* 68, 68–85. <https://doi.org/10.1016/j.ymben.2021.09.004>.
- Novak, K., Neuendorf, C.S., Kofler, I., Kieberger, N., Klamt, S., Pflügl, S., 2021. Blending industrial blast furnace gas with H₂ enables *Acetobacterium woodii* to efficiently co-utilize CO, CO₂ and H₂. *Bioresour. Technol.* 323, 124573. <https://doi.org/10.1016/j.biortech.2020.124573>.
- Olah, G.A., Goepfert, A., Prakash, G.K.S., 2018. Beyond oil and gas: the methanol economy, Third, updated and enlarged edition. Wiley-VCH, Weinheim.
- Öppinger, C., Kremp, F., Müller, V., 2021. Is reduced ferredoxin the physiological electron donor for MetVF-type methylenetetrahydrofolate reductases in acetogenesis? A hypothesis. *Int. Microbiol.* <https://doi.org/10.1007/s10123-021-00190-0>.
- Pacaud, S., Loubiere, P., Goma, G., 1985. Methanol metabolism by *Eubacterium limosum* B2: effects of pH and carbon dioxide on growth and organic acid production. *Curr. Microbiol.* 12, 245–250. <https://doi.org/10.1007/BF01567972>.
- Petrognani, C., Boon, N., Ganigué, R., 2020. Production of isobutyric acid from methanol by *Clostridium luteicellarii*. *Green Chem.* 22, 8389–8402. <https://doi.org/10.1039/D0GC02700F>.
- Poehlein, A., Schmidt, S., Kaster, A.-K., Goenrich, M., Vollmers, J., Thürmer, A., Bertsch, J., Schuchmann, K., Voigt, B., Hecker, M., Daniel, R., Thauer, R.K., Gottschalk, G., Müller, V., 2012. An ancient pathway combining carbon dioxide fixation with the generation and utilization of a sodium ion gradient for ATP synthesis. *PLoS One* 7, e33439. <https://doi.org/10.1371/journal.pone.0033439>.
- Poulalier-Delavalle, M., Baker, J.P., Millard, J., Winzer, K., Minton, N.P., 2023. Endogenous CRISPR/Cas systems for genome engineering in the acetogens

- Acetobacterium woodii and Clostridium autoethanogenum. *Front. Bioeng. Biotechnol.* 11.
- Pregnon, G., Minton, N.P., Soucaille, P., 2022. Genome sequence of Eubacterium limosum B2 and evolution for growth on a mineral medium with methanol and CO₂ as sole carbon sources. *Microorganisms* 10, 1790. <https://doi.org/10.3390/microorganisms10091790>.
- Puiman, L., Abrahamson, B., van der Lans, R.G.J.M., Haringa, C., Noorman, H.J., Picioreanu, C., 2022. Alleviating mass transfer limitations in industrial external-loop syngas-to-ethanol fermentation. *Chem. Eng. Sci.* 259, 117770. <https://doi.org/10.1016/j.ces.2022.117770>.
- Ragsdale, S.W., 2008. Enzymology of the wood–ljungdahl pathway of acetogenesis. *Ann. N. Y. Acad. Sci.* 1125, 129–136. <https://doi.org/10.1196/annals.1419.015>.
- Ragsdale, S.W., Ljungdahl, L.G., 1984. Purification and properties of NAD-dependent 5,10-methylenetetrahydrofolate dehydrogenase from Acetobacterium woodii. *J. Biol. Chem.* 259, 3499–3503. [https://doi.org/10.1016/S0021-9258\(17\)43122-X](https://doi.org/10.1016/S0021-9258(17)43122-X).
- Ragsdale, S.W., Pierce, E., 2008. Acetogenesis and the Wood–Ljungdahl pathway of CO₂ fixation. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1784, 1873–1898. <https://doi.org/10.1016/j.bbapap.2008.08.012>.
- Redl, S., Poehlein, A., Esser, C., Bengelsdorf, F.R., Jensen, T.Ø., Jendresen, C.B., Tindall, B.J., Daniel, R., Dürre, P., Nielsen, A.T., 2020. Genome-based comparison of all species of the genus Moorella, and status of the species Moorella thermoacetica and Moorella thermoautotrophica. *Front. Microbiol.* 10, 3070. <https://doi.org/10.3389/fmicb.2019.03070>.
- Sanford, P.A., Blaby, I., Yoshikuni, Y., Woolston, B.M., 2024. An efficient cre-based workflow for genomic integration and expression of large biosynthetic pathways in Eubacterium limosum. *Biotechnol. Bioeng.* 121, 3360–3366. <https://doi.org/10.1002/bit.28796>.
- Sanford, P.A., Miller, K.G., Hoyt, K.O., Woolston, B.M., 2023. Deletion of biofilm synthesis in Eubacterium limosum ATCC 8486 improves handling and transformation efficiency. *FEMS Microbiol. Lett.* 370, fnad030. <https://doi.org/10.1093/femsle/fnad030>.
- Sanford, P.A., Woolston, B.M., 2024. Development of a recombinering system for the acetogen Eubacterium limosum with Cas9 counterselection for markerless genome engineering. <https://doi.org/10.1101/2024.04.09.588731>.
- Sanford, P.A., Woolston, B.M., 2022. Expanding the genetic engineering toolbox for the metabolically flexible acetogen Eubacterium limosum. *J. Ind. Microbiol. Biotechnol.* kuac019 <https://doi.org/10.1093/jimb/kuac019>.
- Schmidt, S., Biegel, E., Müller, V., 2009. The ins and outs of Na⁺ bioenergetics in Acetobacterium woodii. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1787, 691–696. <https://doi.org/10.1016/j.bbabi.2008.12.015>.
- Schoelmerich, M.C., Müller, V., 2019. Energy conservation by a hydrogenase-dependent chemiosmotic mechanism in an ancient metabolic pathway. *Proc. Natl. Acad. Sci. USA* 116, 6329–6334. <https://doi.org/10.1073/pnas.1818580116>.
- Schrader, J., Schilling, M., Holtmann, D., Sell, D., Filho, M.V., Marx, A., Vorholt, J.A., 2009. Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. *Trends Biotechnol.* 27, 107–115. <https://doi.org/10.1016/j.tibtech.2008.10.009>.
- Schuchmann, K., Müller, V., 2016. Energetics and application of heterotrophy in acetogenic bacteria. *Appl. Environ. Microbiol.* 82, 4056–4069. <https://doi.org/10.1128/AEM.00882-16>.
- Schuchmann, K., Müller, V., 2014. Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. *Nat. Rev. Microbiol.* 12, 809–821. <https://doi.org/10.1038/nrmicro3365>.
- Schuchmann, K., Müller, V., 2013. Direct and reversible hydrogenation of CO₂ to formate by a bacterial carbon dioxide reductase. *Science* 342, 1382–1385. <https://doi.org/10.1126/science.1244758>.
- Schuchmann, K., Müller, V., 2013. Direct and reversible hydrogenation of CO₂ to formate by a bacterial carbon dioxide reductase. *Science* 342, 1382–1385. <https://doi.org/10.1126/science.1244758>.
- Schuchmann, K., Müller, V., 2012. A bacterial electron-bifurcating hydrogenase. *J. Biol. Chem.* 287, 31165–31171. <https://doi.org/10.1074/jbc.M112.395038>.
- Schwarz, F.M., Oswald, F., Müller, V., 2021. Acetogenic conversion of H₂ and CO₂ into formic acid and vice versa in a fed-batch-operated stirred-tank bioreactor. *ACS Sustainable Chem. Eng.* 9, 6810–6820. <https://doi.org/10.1021/acssuschemeng.1c01062>.
- Schwarz, F.M., Schuchmann, K., Müller, V., 2018. Hydrogenation of CO₂ at ambient pressure catalyzed by a highly active thermostable biocatalyst. *Biotechnol. Biofuels* 11, 237. <https://doi.org/10.1186/s13068-018-1236-3>.
- Sharak Genthner, B.R., Bryant, M.P., 1987. Additional characteristics of one-carbon-compound utilization by Eubacterium limosum and Acetobacterium woodii. *Appl. Environ. Microbiol.* 53, 471–476. <https://doi.org/10.1128/aem.53.3.471-476.1987>.
- Shin, J., Bae, J., Lee, H., Kang, S., Jin, S., Song, Y., Cho, S., Cho, B.-K., 2023. Genome-wide CRISPRi screen identifies enhanced autolithotrophic phenotypes in acetogenic bacterium Eubacterium limosum. *Proc. Natl. Acad. Sci.* 120, e2216244120. <https://doi.org/10.1073/pnas.2216244120>.
- Shin, J., Kang, S., Song, Y., Jin, S., Lee, J.S., Lee, J.-K., Kim, D.R., Kim, S.C., Cho, S., Cho, B.-K., 2019. Genome engineering of eubacterium limosum using expanded genetic tools and the CRISPR-Cas9 system. *ACS Synth. Biol.* 8, 2059–2068. <https://doi.org/10.1021/acssynbio.9b00150>.
- Sitara, A., Hocq, R., Horvath, J., Pflügl, S., 2024. Industrial biotechnology goes thermophilic: thermoanaerobes as promising hosts in the circular carbon economy. *Bioresour. Technol.* 408, 131164. <https://doi.org/10.1016/j.biortech.2024.131164>.
- Sitara, A., Hocq, R., Lu, A.J., Pflügl, S., 2025. Hi-TARGET: a fast, efficient and versatile CRISPR type I-B genome editing tool for the thermophilic acetogen Thermoanaerobacter kivui. *Biotechnol. Biofuels Bioprod.* 18, 49. <https://doi.org/10.1186/s13068-025-02647-0>.
- Song, H., Zhang, N., Zhong, C., Liu, Z., Xiao, M., Gai, H., 2017. Hydrogenation of CO₂ into formic acid using a palladium catalyst on chitin. *New J. Chem.* 41, 9170–9177. <https://doi.org/10.1039/C7NJ00460E>.
- Song, Y., Bae, J., Jin, S., Lee, H., Kang, S., Lee, J., Shin, J., Cho, S., Cho, B.-K., 2022. Development of highly characterized genetic bioparts for efficient gene expression in CO₂-fixing Eubacterium limosum. *Metab. Eng.* 72, 215–226. <https://doi.org/10.1016/j.ymben.2022.03.016>.
- Song, Y., Lee, J.S., Shin, J., Lee, G.M., Jin, S., Kang, S., Lee, J.-K., Kim, D.R., Lee, E.Y., Kim, S.C., Cho, S., Kim, D., Cho, B.-K., 2020. Functional cooperation of the glycine synthase-reductase and Wood–Ljungdahl pathways for autotrophic growth of Clostridium drakei. *Proc. Natl. Acad. Sci. U.S.A.* 117, 7516–7523. <https://doi.org/10.1073/pnas.1912289117>.
- Song, Y., Shin, J., Jin, S., Lee, J.-K., Kim, D.R., Kim, S.C., Cho, S., Cho, B.-K., 2018. Genome-scale analysis of syngas fermenting acetogenic bacteria reveals the translational regulation for its autotrophic growth. *BMC Genomics* 19, 837. <https://doi.org/10.1186/s12864-018-5238-0>.
- Strätz, M., Sauer, U., Kuhn, A., Dürre, P., 1994. Plasmid transfer into the homoacetogen Acetobacterium woodii by electroporation and conjugation. *Appl. Environ. Microbiol.* 60, 1033–1037. <https://doi.org/10.1128/AEM.60.3.1033-1037.1994>.
- Straub, M., Demler, M., Weuster-Botz, D., Dürre, P., 2014. Selective enhancement of autotrophic acetate production with genetically modified Acetobacterium woodii. *J. Biotechnol.* 178, 67–72. <https://doi.org/10.1016/j.jbiotec.2014.03.005>.
- Strong, P.J., Xie, S., Clarke, W.P., 2015. Methane as a resource: can the methanotrophs add value? *Environ. Sci. Technol.* 49, 4001–4018. <https://doi.org/10.1021/es504242n>.
- Thauer, R.K., Kaster, A.-K., Seedorf, H., Buckel, W., Hedderich, R., 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* 6, 579–591. <https://doi.org/10.1038/nrmicro1931>.
- Tremblay, P.-L., Höglund, D., Koza, A., Bonde, I., Zhang, T., 2015. Adaptation of the autotrophic acetogen Sporomusa ovata to methanol accelerates the conversion of CO₂ to organic products. *Sci. Rep.* 5, 16168. <https://doi.org/10.1038/srep16168>.
- Tremblay, P.-L., Zhang, T., 2023. Genetic tools for the electrotoph Sporomusa ovata and autotrophic biosynthesis. *Appl. Environ. Microbiol.* 90, e01757-23. <https://doi.org/10.1128/aem.01757-23>.
- Valgepea, K., de Souza Pinto Lemgruber, R., Meaghan, K., Palfreyman, R.W., Abdalla, T., Heijstra, B.D., Behrendorf, J.B., Tappel, R., Köpke, M., Simpson, S.D., Nielsen, L.K., Marcellin, E., 2017. Maintenance of ATP homeostasis triggers metabolic shifts in gas-fermenting acetogens. *Cell Syst.* 4, 505–515.e5. <https://doi.org/10.1016/j.cels.2017.04.008>.
- Van Dien, S., 2013. From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. *Curr. Opin. Biotechnol.* 24, 1061–1068. <https://doi.org/10.1016/j.copbio.2013.03.002>.
- Vom Berg, C., Carus, M., Stratmann, M., Dammer, L., 2022. Renewable Carbon as a Guiding Principle for Sustainable Carbon Cycles. *Renewable Carbon Initiative (RCI)*. <https://doi.org/10.52548/QUHG1295>.
- Wang, J., Liao, Y., Qin, J., Ma, C., Jin, Y., Wang, X., Chen, K., Ouyang, P., 2023. Increasing lysine level improved methanol assimilation toward butyric acid production in Butyrobacterium methylotrophicum. *Biotechnol. Biofuels Bioproducts* 16, 10. <https://doi.org/10.1186/s13068-023-02263-w>.
- Wang, X., Qin, J., Ma, C., Wang, J., Wang, X., Xu, S., Feng, J., Chen, K., Ouyang, P., 2021. Methanol assimilation with CO₂ reduction in butyrobacterium methylotrophicum and development of genetic toolkits for its engineering. *ACS Sustainable Chem. Eng.* 9, 12079–12090. <https://doi.org/10.1021/acssuschemeng.1c02365>.
- Wang, Y., Dong, M., Li, S., Chen, B., Liu, H., Han, B., 2024. The superiority of Pd²⁺ in CO₂ hydrogenation to formic acid. *Chem. Sci.* 15, 5525–5530. <https://doi.org/10.1039/D3SC006925G>.
- Westphal, L., Wiechmann, A., Baker, J., Minton, N.P., Müller, V., 2018. The Rnf complex is an energy-coupled transhydrogenase essential to reversibly link cellular NADH and ferredoxin pools in the acetogen Acetobacterium woodii. *J. Bacteriol.* 200. <https://doi.org/10.1128/JB.00357-18>.
- Wirth, S., Dürre, P., 2021. Investigation of putative genes for the production of medium-chained acids and alcohols in autotrophic acetogenic bacteria. *Metab. Eng.* 66, 296–307. <https://doi.org/10.1016/j.ymben.2021.04.010>.
- Wood, H.G., 1991. Life with CO or CO₂ and H₂ as a source of carbon and energy. *FASEB J.* 5, 156–163. <https://doi.org/10.1096/fasebj.5.2.1900793>.
- Wood, J.C., Gonzalez-Garcia, R.A., Daygon, D., Talbo, G., Plan, M.R., Marcellin, E., Virdis, B., 2023a. Characterisation of acetogen formatotrophic potential using Eubacterium limosum. *Appl. Microbiol. Biotechnol.* <https://doi.org/10.1007/s00253-023-12600-6>.
- Wood, J.C., Gonzalez-Garcia, R.A., Daygon, D., Talbo, G., Plan, M.R., Marcellin, E., Virdis, B., 2023b. Molecular understanding of Eubacterium limosum chemostat methanol metabolism. *Sustainable Energy Fuels* 7, 2397–2412. <https://doi.org/10.1039/D2SE01551J>.
- Wood, J.C., Marcellin, E., Plan, M.R., Virdis, B., 2021. High methanol-to-formate ratios induce butanol production in Eubacterium limosum. *Microb. Biotechnol.* <https://doi.org/10.1111/1751-7915.13963>.
- Xiao, Y., Zhang, E., Zhang, J., Dai, Y., Yang, Z., Christensen, H.E.M., Ulstrup, J., Zhao, F., 2017. Extracellular polymeric substances are transient media for microbial extracellular electron transfer. *Sci. Adv.* 3, e1700623. <https://doi.org/10.1126/sciadv.1700623>.

- Yishai, O., Lindner, S.N., Gonzalez De La Cruz, J., Tenenboim, H., Bar-Even, A., 2016. The formate bio-economy. *Curr. Opin. Chem. Biol.* 35, 1–9. <https://doi.org/10.1016/j.cbpa.2016.07.005>.
- Yu, J., Park, M.-J., Lee, J., Kwon, S.J., Lim, J.K., Lee, H.S., Kang, S.G., Lee, J.-H., Kwon, K.K., Kim, Y.J., 2023. Genomic potential and physiological characteristics of C1 metabolism in novel acetogenic bacteria. *Front. Microbiol.* 14.
- Zeikus, J.G., Lynd, L.H., Thompson, T.E., Krzycki, J.A., Weimer, P.J., Hegge, P.W., 1980. Isolation and characterization of a new, methylotrophic, acidogenic anaerobe, the marburg strain. *Curr. Microbiol.* 3, 381–386. <https://doi.org/10.1007/BF02601907>.