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MINI REVIEW

Leveraging substrate flexibility and product selectivity of acetogens in two-stage systems for chemical production

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Abstract

Carbon dioxide (CO_2) stands out as sustainable feedstock for developing a circular carbon economy whose energy supply could be obtained by boosting the production of clean hydrogen from renewable electricity. H_2-dependent CO_2 gas fermentation using acetogenic microorganisms offers a viable solution of increasingly demonstrated value. While gas fermentation advances to achieve commercial process scalability, which is currently limited to a few products such as acetate and ethanol, it is worth taking the best of the current state-of-the-art technology by its integration within innovative bioconversion schemes. This review presents multiple scenarios where gas fermentation by acetogens integrate into double-stage biotechnological production processes that use CO_2 as sole carbon feedstock and H_2 as energy carrier for products' synthesis. In the integration schemes here reviewed, the first stage can be biotic or abiotic while the second stage is biotic. When the first stage is biotic, acetogens act as a biological platform to generate chemical intermediates such as acetate, formate and ethanol that become substrates for a second fermentation stage. This approach holds the potential to enhance process titre/rate/yield metrics and products' spectrum. Alternatively, when the first stage is abiotic, the integrated two-stage scheme foresees, in the first stage, the catalytic transformation of CO_2 into C_1 products that, in the second stage, can be metabolized by acetogens. This latter scheme leverages the metabolic flexibility of acetogens in efficient utilization of the products of CO_2 abiotic hydrogenation, namely formate and methanol, to synthesize multicarbon compounds but also to act as flexible catalysts for hydrogen storage or production.

INTRODUCTION

With growing alarms about global grave climate changes and increasing demand for sustainable production schemes, it becomes clear that we need to drift ourselves from our dependency on fossil carbons and redefine our production and consumption patterns (Kümmerer et al., 2020). Ultimately, the only truly sustainable carbon feedstock for a circular carbon economy is carbon dioxide (CO_2), with hydrogen (H_2) deemed as the enabler of the lowest-cost low-carbon energy system (van der Spek et al., 2022; van Renssen, 2020). The first supplies the elemental carbon, while the second provides the energy for converting CO_2 into useful products.

Among the natural CO_2 fixation pathways, the Wood–Ljungdahl (or reductive acetyl-CoA) pathway (WLP) found in anaerobic acetogens is particularly efficient (Fast &

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Papoutsakis, 2018; Ragsdale & Pierce, 2008). Until around a decade ago, concerns around genetic inaccessibility, energetics and process scale-up stood as major hurdles before the adoption of gas fermentation as a commercial platform (Köpke et al., 2011). Over the past decade, an enhanced understanding of key pathways for autotrophic growth and their regulatory aspects of energy conservation as well as of carbon distribution and electron balancing allowed to address several of the open questions regarding the genetic and metabolic challenges of C_1-gas fermenting acetogens (Di Leonardo et al., 2022; Pavan et al., 2022, Schuchmann & Müller, 2014). Experimental and computational system-level analyses of a growing number of gas-fermenting processes supported a better understanding of cellular behaviour and of its application in biocatalytic systems' development (de Lima et al., 2022; Ghadermazi et al., 2022; Mahamkali et al., 2020; Molitor et al., 2017; Valgepea et al., 2017). Additionally, several studies focused on the development of efficient reactor configurations combining enhanced gas-to-liquid mass transfer with low power consumption (Asimakopoulos et al., 2018; Elisiario et al., 2022; Stoll et al., 2019; Takors et al., 2018). Over the past several years, synthetic biology approaches have been employed to develop acetogens in efficient platform strains for C_1 gas conversion, focusing in particular on the manipulation of metabolic fluxes aimed at the production of non-native compounds (Bourgade et al., 2021; Lee et al., 2022). Intense process optimizations along with pilot- and demonstration-plants operations addressed the at-scale operability of the application of acetogens in gas fermentation processes (Fackler et al., 2021; Liew et al., 2022). Acetogens have already found commercial deployment to reduce CO_2 using H_2 as energy source and produce biofuels and commodities, mainly ethanol and acetate (Köpke & Simpson, 2020).

The emergence of established gas fermentation systems paves the way to manifold options for their inclusion in integrated carbon circular biorefineries where multiple processing units can operate in cascade. Combining the high substrate flexibility and product selectivity advantages featured by acetogenic bacteria with the product diversity options of aerobic systems within integrated biotechnological routes lends noticeable advantages such as the increase in achievable titres and productivities, and the widening of the affordable products' portfolio. Indeed, due to the anaerobic life style, acetogenic bacteria are energy limited and the production of long-chain or ATP-demanding molecules is challenging. At least four different strategies are in place to broaden the fields of application of gas fermentation with acetogens: (1) genetic modification of a pure culture in a single bioprocess stage; (2) use of co-cultures in a single bioprocess stage; (3) use of an undefined mixed culture in a single bioprocess stage and (4) realization of separate bioprocessing stages with pure cultures. This review offers a concise overview of current developments and future prospects about the integration of acetogenic pure cultures into double-stage biotechnological production paths using CO_2 and H_2 for products' synthesis. We focus exclusively on two-stage processes such that: (a) the gas substrates in the first stage (independently on whether the first stage is abiotic or biotic) is exclusively a mixture of CO_2 and H_2, (b) the first stage converts the gas substrates CO_2/H_2 into products that, in the second stage, become the substrates for microbial production of an ample spectrum of value-added chemicals by a pure culture, (c) the first stage converting CO_2/H_2 into the intermediate products can rely either on a catalytic process or on a gas fermentation process operated by an acetogenic culture, (d) at least one of the two stages is reliant on an acetogenic culture and (e) when acetogens are used in the first stage to start the double-stage process, the spectrum of gas fermentation products considered does not account for non-native products but is limited to the native products acetate, ethanol and formate. We show chief advantages of such two-stage processes include not only advantages directly related to the optimal development of the single catalysts employed in the separate stages but encompass also advantages strictly related to the integrated design and operation of the technological equipment such as modularity, controllability, circularity and infrastructural attractiveness. Throughout our review, we emphasize the steps where the role of acetogenic bacteria has already been proven or can be envisaged.

OPPORTUNITIES IN PROCESS INTEGRATION

Multi-stage fermentations consist of distinct and interlinked technological processes where the products derived from one process stage are the substrates for the process that takes place in the subsequent stage. Within the scope of this review, the multiple-stage process routes can be entirely biological (all conversions are biotic) or hybrid (conversions can be biotic or abiotic). Figures 1 and 2 displays multiple typifying scenarios where, owing to their substrates' flexibility and products' selectivity, acetogenic bacteria are ideal biocatalysts to carry out one of the stages of the processes in cascade.

BIOLOGICAL TWO-STAGE PROCESSES FOR PRODUCTS' SYNTHESIS FROM CARBON DIOXIDE AND HYDROGEN

Two-stage biotic processes through the acetate intermediate

According to the integrated two-stage process proposed in Figure 1, in a first step, a gas mixture consisting of CO_2 and H_2 serves as carbon and energy

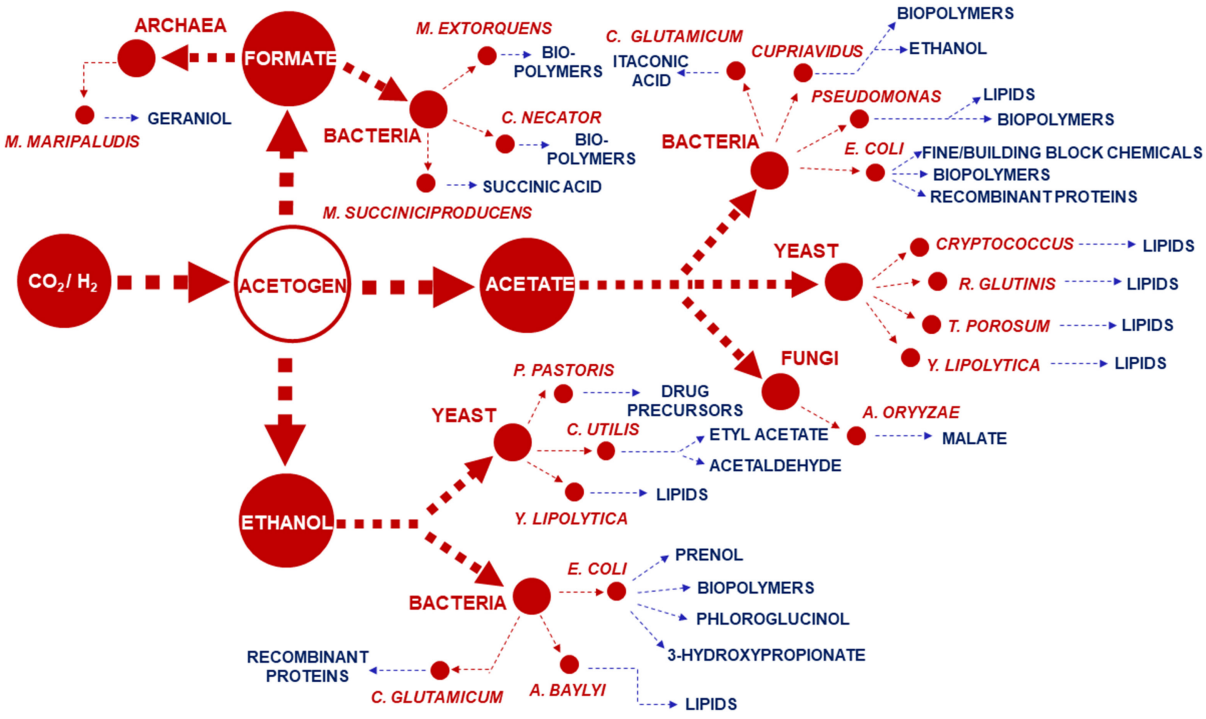

FIGURE 1 Biological two-stage processes for products' synthesis from carbon dioxide and hydrogen. The figure shows different scenarios of two-stage bioprocessing systems based on H_2-dependent CO_2 fermentation using acetogens. Acetogens act as platform organisms for the production of acetate, ethanol and formate that in turn can be assimilated by a variety of organisms to achieve biotechnologically relevant compounds. Production strains are displayed in red whereas products in blue. Literature references underlying the depicted end-products are thoroughly reported in Table 1. *M. Maripaludis, Methanococcus Maripaludis; M. succiniciproducens, Mannheimia succiniciproducens; M. extorquens, Methylorubrum extorquens; C. necator, Cupriavidus necator; C. glutamicum, Corynebacterium glutamicum; R. glutinis, Rhodotorula glutinis; T. porosum, Trichosporon porosum; Y. lipolytica, Yarrowia lipolytica; P. pastoris, Pichia pastoris; C. utilis, Candida utilis; A. baylyi, Acinetobacter baylyi.*

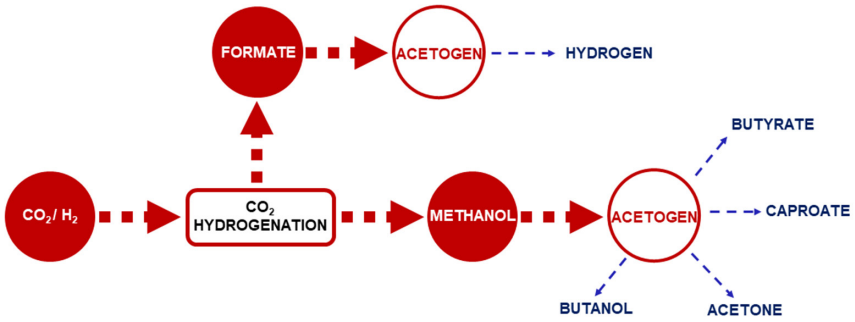

FIGURE 2 Two-stage processes using products of abiotic carbon dioxide hydrogenation as liquid substrates for acetogenic cultures. The figure shows exemplary scenarios based on the abiotic production of the intermediates methanol and formate by CO_2 hydrogenation. Both methanol and formate can be used as substrates for acetogenic cultures to produce several multicarbon compounds and hydrogen.

source for autotrophic acetogenic bacteria to generate acetate. In a second step, acetate is further valorized by acetate-converting microorganisms of industrial biotechnology, including both established hosts such as *Escherichia coli*, *Corynebacterium glutamicum* and oleaginous yeasts but also unconventional prokaryotic hosts (Blombach et al., 2022; Kiefer, Merkel, Lilge, Henkel, & Hausmann, 2021), to produce a variety of bio-based products including building block chemicals, microbial lipids and biopolymers (Kiefer, Merkel, Lilge, Henkel, & Hausmann, 2021). The point of merit of linking distinct microbial fermentations in a cascaded process is the opportunity of greatly expanding the spectrum of biotechnologically relevant products in which we could profitably recycle the cumbersome waste CO_2. The key characteristic of acetogens that is exploited in this scenario is the ability to efficiently assimilate CO_2 and to convert it in a chemical intermediate – acetate – whose assimilation by microorganisms is considerably easier and more efficient than that of CO_2 (Ma et al., 2022). Acetate is the predominant product if only CO_2 and H_2 are available as substrates for the acetogenic culture

(Demler & Weuster-Botz, 2011; Ricci et al., 2021; Schiel-Bengelsdorf & Dürre, 2012). The highest acetate titre of 59.2 gL^{-1} reported so far in literature was achieved during gas fermentation of *Acetobacterium woodii* (*A. woodii*) DSM 1030 using a CO_2/H_2 gas mixture (Kantzow et al., 2015). The potential of the two-stage approach lies in the extremely high versatility of acetate as substrate in industrial biotechnology (Kiefer, Merkel, Lilge, Henkel, & Hausmann, 2021; Kim et al., 2021). For instance, using acetate as substrate for several engineered *E. coli* strains led to the generation of several products, such as free fatty acids, isopropanol, succinate, isobutanol, 3-hydroxypropionic acid, itaconic acid and 2,3-butanediol, as reviewed by Kutscha and Pflügl (2020) and Kiefer, Merkel, Lilge, Henkel, and Hausmann (2021). Table 1 summarizes representative examples where acetate can be microbiologically upgraded into valuable compounds.

Integrated two-stage processes, in which acetate produced by acetogenic CO_2/H_2 gas fermentation is further converted into bioproducts, has already achieved considerable progress at the proof-of-concept or lab-scale level. For instance, (Molitor et al., 2019) introduced a two-stage bioprocessing system consisting of a first stage with a pure culture of *Clostridium ljungdahlii* (*C. ljungdahlii*) to produce acetate from CO_2 and H_2, and a second stage with a pure culture of *Saccharomyces cerevisiae* to convert acetate with oxygen and a nitrogen source into single-cell-protein (SCP). This two-stage bioprocessing system is promising even though considerable technical optimizations are necessary to reach industrially relevant protein production rates of approximately 1 gL^{-1} h^{-1}, which is a 14 times increase from this proof-of-concept study (Molitor et al., 2019).

One of the most interesting products achieved so far by the proposed two-stage bioprocess concept is polyhydroxybutyrate (PHB), a thermoplastic polyester accumulated by various microorganisms as intracellular storage material, in response to stressful growth conditions (Choi et al., 2020). PHB is drawing commercial attention since it is poised to become an excellent candidate to substitute petroleum-derived plastics in several applications such as food packaging and medicine (Hatti-Kaul et al., 2020; Turco et al., 2021). In Al Rowaihi et al. (2018), the acetic acid (3.2 gL^{-1}) generated by *A. woodii* from CO_2/H_2 gas mixture was converted by *Ralstonia eutropha* H16 to PHB (0.5 gL^{-1} PHB, q_{PHB} of 98.4 mg_{PHB} L^{-1} h^{-1} and PHB content defined as the ratio of PHB concentration to cell concentration of around 33%). Carefully evaluated aspects to setup the integrated bioprocess included the increase in the gas-to-liquid mass transfer by applying high-pressure conditions without excessive gas loss in the first stage, and the usage of a single medium that only required pH adjustment depending on the bioprocess stage. The two-stage bioprocess developed in Cestellos-Blanco et al. (2021) showed a peak of acetate production of 10.4 mmol acetate $L^{-1}d^{-1}$ from CO_2 by *Sporumosa ovata*, which subsequently translated into 12.54 mg_{PHB} L^{-1} h^{-1} by *Cupriavidus basilensis* in the unprocessed media with an overall carbon yield of 11.06% from acetate. In this case, the production of PHB from CO_2 occurred with limited intermediate purification and processing steps but can expectedly improve by undertaking further optimizations of each biocatalyst step (Sohn et al., 2021). According to the data reported in ref. (Al Rowaihi et al., 2018) and in ref. (Cestellos-Blanco et al., 2021), the bioprocessing systems developed so far afforded the storage of 21.6% and 29.2% of the carbon in the CO_2 substrate in the PHB product (Table 2).

A thematically aligned process flow comprising a two-stage system was applied also for the production of lipids (Hu et al., 2016). First, an anaerobic bubble-column bioreactor converted gas mixtures to acetic acid, using the anaerobic acetogen *Moorella thermoacetica*. Second, the produced acetic acid was fed as a substrate to a second stirred-tank bioreactor, where it was converted aerobically into lipids by an engineered oleaginous yeast, *Yarrowia lipolytica*. The integrated continuous bench-scale reactor system produced 18 g/L of C_{16}–C_{18} triacylglycerides from gas, with an overall productivity of 0.19 gL^{-1} h^{-1} and a lipid content of 36%. Here, it should be remarked that an important part of the process required for achieving higher overall acetate productivity was the gas composition switch strategy. Indeed, *M. thermoacetica* was first grown on a CO/CO_2 mixture to establish the culture and then switched to a CO_2/H_2 mixture to take advantage of the higher acetate specific productivity on hydrogen.

Bioprocesses for fermentation of C_1 gases into value-added chemicals through the acetate intermediate have gained increased interest from the industrial sector. For instance, the biotech company LanzaTech Inc. (Illinois, USA) started a cooperation with the Malaysian oil and gas company Petronas (Kuala Lumpur, Malaysia) in 2012 to produce acetate from CO_2-containing gases from several sources like refinery off gases and natural gas wells. In 2019, IndianOil Ltd. (New Delhi, India) announced the construction of a commercial-scale production plant for microbial lipid production from low-cost CO_2-generated acetate (Kiefer, Merkel, Lilge, Henkel, & Hausmann, 2021). Thus, two-stage bioprocessing routes comprising fermentation of low-cost C_1 gases into acetate as low-cost carbon source for the final bioproduction stage may probably become a remarkable route with industrial competitiveness in the near future.

Two-stage biotic processes through the ethanol intermediate

When grown on CO_2 and H_2 only, acetogenic cultures also produce ethanol in substantial amount. Ethanol

TABLE 1 Bioproducts obtained by biotic two-stage processes using CO_2/H_2 as gaseous substrates for acetogenic bacteria.

	2nd stage				
1st stage product		**Strain**	**Strain growth reference**	**Product**	**Product reference**
acetate	Bacteria	*Escherichia coli*	Oh et al., 2002	Acetone, itaconic acid, isobutanol, isopropanol, mevalonic acid, phloroglucinol biopolymers, succinic acid, tyrosine, ß-caryophyllene, 2,3-butanediol, recombinant proteins	Chen et al., 2018; Henkel, & Hausmann, 2021; Kiefer, Merkel, Lilge, Novak et al., 2020; Leone et al., 2015
		Corynebacterium glutamicum	Arnold, Henkel, et al., 2019; Gerstmeir et al., 2003; Jolkver et al., 2009	Itaconic acid	Kiefer, Merkel, Lilge, Hausmann, & Henkel, 2021; Merkel et al., 2022
		Pseudomonas aeruginosa	Dolan et al., 2020	Biopolymers	Saito & Doi, 1993
		Pseudomonas putida	Arnold, Tews, et al., 2019; Arias-Barrau et al., 2006	Biopolymers; rhamnolipids	Arnold, Henkel, et al., 2019; Yang et al., 2019
		Cupriavidus basilensis	Wierckx et al., 2010	Biopolymers	Cestellos-Blanco et al., 2021
		Cupriavidus necator	Marudkla et al., 2018	Biopolymers; ethanol	Lee et al., 2016; Marudkla et al., 2018
		Cobetia sp. MC34 and Cobetia marina DSM 4741T	Christensen et al., 2021	Biopolymers	Christensen et al., 2021
		Rhodobacter sphaeroides	Alber et al., 2006	Hydrogen	Shimizu et al., 2019, 2022
		Rhodobacter capsulatus	Willison, 1988	Hydrogen	Gürgan et al., 2015
	Yeasts	*Cryptococcus curvatus*	Liu et al., 2017	Lipids	Gong et al., 2015
		Cryptococcus podzolicus	Qian et al., 2020	Lipids	Qian et al., 2020
		Rhodotorula glutinis	Kolouchová et al., 2015	Lipids	Zhang et al., 2019
		Yarrowia lipolytica	Spagnuolo et al., 2018	Lipids	Chen et al., 2021; Qiao et al., 2017
		Trichosporon porosum	Qian et al., 2020	Lipids	Qian et al., 2020
		Kluyveromyces polysporus	Kolouchová et al., 2015	Lipids	Kolouchová et al., 2015
		Saccharomyces cerevisiae	Kolouchová et al., 2015	Lipids	Kolouchová et al., 2015
		Torulaspora delbrueckii	Kolouchová et al., 2015	Lipids	Kolouchová et al., 2015
	Fungi	*Aspergillus oryzae*	Oswald et al., 2016	Malic acid	Oswald et al., 2016
	Archaea	*Methanosarcina acetivorans*	Ferry, 2020	Methane	Ferry, 2020
		Methanothrix thermophila	Inatomi et al., 1993	Methane	Inatomi et al., 1993

TABLE 1 (Continued)

1st stage product	2nd stage	Strain	Strain growth reference	Product	Product reference
Ethanol	Bacteria	*Pseudomonas aeruginosa*	Bosire et al., 2016; Kretzschmar et al., 2001	Trehalose; rhamnolipids, biopolymers	Harty et al., 2019; Hori et al., 2002
		Pseudomonas putida	Sarwar et al., 2022	Fatty acid ethyl esters	Sarwar et al., 2022
		E. coli	Liang et al., 2021	Biopolymers, prenol, phloroglucinol, 3-hydroxypropionate	Liang et al., 2021; Sun et al., 2020
		Corynebacterium glutamicum	Arndt et al., 2008	Recombinant proteins	Yu et al., 2022
	Yeasts	*Pichia pastoris*	Liu et al., 2019	Monacolin J; recombinant proteins	Ergün et al., 2019; Liu et al., 2019
		Candida utilis	Domenech et al., 2004	Ethyl acetate; acetaldehyde	Domenech, 2004
		Yarrowia lipolytica	Gatter et al., 2016	Alpha-ketoglutaric acid	Chernyavskaya et al., 2000
Formate	Bacteria	*E. coli*	Delmas et al., 2022; Kim et al., 2020	Pyruvate	Kirst et al., 2022
		Cupriavidus necator	Claassens et al., 2020; Grunwald et al., 2015	Biopolymers	Stöckl et al., 2020
		Mannheimia succiniciproducens	Ahn et al., 2017	Succinic acid	Ahn et al., 2017
		Methylorubrum extorquens	Chang et al., 2022	Biopolymers	Chang et al., 2022
	Archaea	*Methanococcus maripaludis*	Lyu et al., 2016	Geraniol	Lyu et al., 2016

Note: The table reports representative examples of possible two-stage processes using the acetate, ethanol, or formate produced by CO_2-H_2 grown acetogens as chemical intermediates for microbial conversion into bioproducts. The table displays microorganisms known to be able to grow on each of the aforementioned substrate and reports examples of products that could be obtained. References in support of growth evidence and of product formation are included in the table.

TABLE 2 Carbon and energy balances corresponding to PHB production in a two-stage process where the intermediate is either acetate or ethanol derived from CO_2/H_2 fermentation

	Acetate	-->	Biomass	PHB	Energy balance [kJ]	Energy yield PHB [%]	Energy yield 1st stage [%]	Total energy yield 2 stages [%]	Carbon yield 1st stage [%]	Total carbon yield 2 stages [%]
PHB (Al Rowaihi et al., 2018; Hermann et al., 2020)										
Molar formula	$C_2O_2H_4$	-->	$CH_2O_{0.5}$	$C_4H_6O_2$						
Reaction stoichiometry [moles]	1	-->	0.32	0.12						
Energy content [kJ/mol]	802.4	-->	529.7	1950						
Energy balance [kJ]	802.4	-->	169.5	226.2	406.7	28.19	75.76	21.36	93	21.58
Reaction stoichiometry [C-moles]	1	-->	0.1600	0.2320						
PHB (Cestellos-Blanco et al., 2021; Hermann et al., 2020)										
Molar formula	$C_2O_2H_4$	-->	$CH_2O_{0.5}$	$C_4H_6O_2$						
Reaction stoichiometry [moles]	1	-->	0.94	0.16						
Energy content [kJ/mol]	802.4	-->	529.66	1950						
Energy balance [kJ]	802.4	-->	495.76	306.2	0.4757	38.16	75.76	28.91	93.00	29.20
Reaction stoichiometry [C-moles]	1	-->	0.47	0.3140						
PHB (Hermann et al., 2020; Sun et al., 2020)										
Molar formula	C_2H_6O	-->	$CH_2O_{0.5}$	$C_4H_6O_2$						
Reaction stoichiometry [moles]	1	-->	1.21	0.17						
Energy content [kJ/mol]	1233.4	-->	529.7	1950						
Energy balance [kJ]	1233.4	-->	640.4	333.8	261.5	27.06	54.57	14.77	54.50	18.66
Reaction stoichiometry [C-moles]	1	-->	0.6045	0.3423						

Note: CO_2/H_2 fermentation taking place in the first stage is assumed to produce solely ethanol or acetate, each of which is then assumed to act as substrate in the second stage. To assemble the carbon and energy balances, we gathered available information on PHB production from acetate with *R. eutropha* H16 (Al Rowaihi et al., 2018), PHB production from acetate with *C. basilensis* (Cestellos-Blanco et al., 2021), PHB production from ethanol with *E. coli* Q3094 (Sun et al., 2020), and acetate/ethanol production from H_2/CO_2 with *C. ljungdahlii* (Hermann et al., 2020). Two-stage energy and carbon yields were obtained by multiplying the yields of the two respective stages. Details on the computation are provided in the main text and Table S1.

itself can be microbially upgraded (Zhang et al., 2022). As ethanol can be directly converted into acetyl-CoA, it is suitable to produce acetyl-CoA derivatives (Ma et al., 2022). It is plausible to envisage two-stage bioprocessing systems where the acetogenic fermentation provides ethanol as carbon feedstock for the subsequent bioproduction stage carried out by ethanol-catalysing chassis. Until now, the most successful example of gas fermentation-based ethanol valorization is microbial chain elongation in which ethanol acts as energy and carbon source to elongate short-chain carboxylic acids to longer-chain ones (typically C_4–C_8). Chain elongation usually makes use of open cultures of microbial consortia although recently a pure culture of *Clostridium kluyveri* (*C. kluyveri*) was used in continuous bioreactors to convert ethanol/acetic acid mixtures into medium-chain carboxylic acids (Gildemyn et al., 2017). A single-stage process proved the feasibility of converting ethanol resulting from syngas fermentation to carboxylates by means of a co-culture of *C. ljungdahlii* and *C. kluyveri* (Richter et al., 2016). However, this study pointed out the challenges of identifying an operational space suitable for both members of the co-culture, and highlighted the low specificity of the products, which originates from the conversion of the produced carboxylates to their corresponding alcohols. On the other side, two-stage processes afford enhanced controllability of process conditions and allow optimizing the ethanol/acetic acid ratio for further chain elongation in the second stage. Beyond the benefits in carboxylate chain elongation, two-stage processes foreseeing ethanol as the intermediate deserve attention with the aim of further expanding the spectrum of products achievable from CO_2/H_2.

There are several examples of microorganisms assimilating ethanol and converting ethanol in chemicals. A recent study highlighted the biotechnological potential of the Crabtree-negative yeast *Pichia pastoris*, which was engineered to utilize ethanol as sole carbon source for cell growth and production of a key intermediate – monacolin J – of a semi-synthetic cholesterol-lowering drug, simvastatin (Liu et al., 2019). Further chemicals produced using ethanol as the sole carbon source include two acetyl-CoA derivatives, the biopolymer PHB and the terpenoid prenol. With a metabolic engineering approach, the engineered *E. coli* strain grew on ethanol as the sole carbon source and produced 0.6 g/L of PHB from 10 g/L of ethanol in 96 h and 24 mg/L of prenol from 10 g/L of ethanol in 48 h (Liang et al., 2021). Another study recently explored whether the bioconversion of ethanol into acetyl-CoA-derived chemicals such as phloroglucinol and 3-hydroxypropionate is achievable in recombinant *E. coli* strains, and gathered positive results (Sun et al., 2020). As shown in these studies, deploying heterologous ethanol utilization pathway in microbial hosts proposes itself as biotechnological tool to produce value-added acetyl-CoA derived chemicals. With further strain and process development, the CO_2-derived ethanol may become an abundant, renewable, and affordable substrate to fuel ethanol-based fermentation processes (Table 1).

Based on our survey of acetate- and ethanol-assimilating chassis organisms, acetate and ethanol could be in some cases alternative substrates to support the microbial production of a certain target compound in the second stage of a two-stage process. We would like to remark the relevance of carefully analysing under which conditions a certain two-stage fermentation process represents a viable option to pursue. Since PHB, one of the products obtainable from ethanol, was also produced from acetate in purposely engineered *E. coli* strains (Sun et al., 2020), we found it interesting to compare the hypothetical PHB yields that could be obtained assuming that the PHB bioprocessing step is in cascade to a CO_2/H_2 gas fermentation process producing solely acetate or ethanol. Since we found no evidence of the realization of a similar two-stage process foreseeing ethanol as intermediate, we set out to rely on individual studies to obtain quantitative data separately on the single stages and to tentatively forecast the PHB yields in the hypothesized double-stage processes. In particular, we extracted the data useful for quantifying PHB production from ethanol in ref. (Sun et al., 2020) and from acetate in ref. (Cestellos-Blanco et al., 2021) and in ref. (Al Rowaihi et al., 2018). Similarly, we gathered quantitated information on acetate and ethanol production using acetogenic cultures grown on CO_2-H_2 from ref. (Hermann et al., 2020). PHB derives from acetyl-CoA by condensing two acetyl-CoA molecules to one acetoacetyl-CoA that is reduced and subsequently polymerized. Acetate and ethanol can be directly converted into acetyl-CoA. Acetyl-CoA is produced from acetate via two different pathways, which are catalysed, respectively, by the AMP-forming acetyl-CoA synthetase (ACS) and the phsphotransacetylase/acetate kinase (Pta-AckA). Each of these routes consumes ATP for the production of acetyl-CoA from acetate, and does not produce any reducing power, suggesting that additional acetate is required to generate ATP and reducing power when acetate is the sole carbon source. On the other hand, the transformation of ethanol into acetyl-CoA generates two NADH per ethanol for ATP regeneration, which reduces the need of oxidizing acetyl-CoA for harvesting energy and thus is expected to lead to higher yields of acetyl-CoA-derived chemicals such as PHB (Liang et al., 2021; Sun et al., 2020). Nonetheless, in the two-stage scenario envisaged here, where ethanol or acetate are supposed to derive from a CO_2/H_2 gas fermentation bioprocess, we have to account for the fact that more hydrogen is required to reduce CO_2 to ethanol than to acetate. Indeed, when we evaluated the hypothesized two-stage processes, ethanol did not seem to outperform acetate as substrate for PHB production.

As recapitulated in Table S1, the yields of PHB from H_2 and CO_2 when ethanol is the sole intermediate in the two-stage process are comparable or slightly lower than the yields obtained when acetate is the sole intermediate (with slight variations depending on the study used to quantitate the PHB production from acetate). With no intent to be conclusive on the particular two-stage process discussed here, we employed the case study to warn about the risk of automatically drawing conclusions on processes' combination just on the basis of the advantages brought about by the individual processes.

Two-stage biotic processes through the formate intermediate

Formate is drawing great attention as one of the simplest organic compounds for providing both carbon and energy to microorganisms for bioproduction processes (Ahn et al., 2017; Chang et al., 2022; Kirst et al., 2022; Lyu et al., 2016; Yishai et al., 2016). Table 1 provides representative examples. Naturally occurring formate-assimilation pathways have been introduced or enhanced into industrial workhorses, such as *E. coli, Cupriavidus necator* and yeasts, by genetic rewiring (Claassens, 2021; Yishai et al., 2018) and laboratory evolution strategies (Kim et al., 2020). Furthermore, synthetic formate-fixing pathways have recently been introduced (Bang et al., 2021; Bar-Even, 2016) such that different host organisms, cultivation conditions and desired products could be matched with the most suitable pathway (Bar-Even, 2016; Qiao et al., 2021). The introduction of synthetic or natural formate assimilation pathways in model organisms such as *E. coli* allows to couple the formate assimilation capability with the potential to biosynthesize a vast products' portfolio by exploiting the unparalleled toolbox that is available for biocatalytic systems construction in model organisms. Therefore, the capability of acetogens to produce formate starting from CO_2/H_2 is an interesting trait to develop two-stage bioproduction systems. In this scenario, acetogens can be recruited in the first step to produce formate, which in turn becomes the substrate used, in the second step, by natural or synthetic formate-metabolizing biocatalysts to produce multicarbon compounds. Several formate-producing paths have already been suggested and tested using acetogenic cultures. One of these strategies foresaw increasing the absolute system pressure of acetogenic cultivations (Kantzow & Weuster-Botz, 2016; Oswald et al., 2018). For instance, the pressurization of a *C. ljungdahlii* culture in *a* batch stirred tank reactor resulted in a shift of the product spectrum in favour of formic acid. Indeed, formic acid became the predominant product at a total pressure of 7 bar, with 0.8 g/L acetic acid and 3.2 gL^{-1} formic acid produced over the course of fermentation (Oswald et al., 2018). Increased formic acid production at elevated pressures with CO_2/H_2 is described also by (Kantzow & Weuster-Botz, 2016) for *A. woodii*. While this approach is worth of investigation, the turnaround technology for efficient biological CO_2 hydrogenation operations in acetogens was represented by the discovery of a bacterial hydrogen-dependent carbon dioxide reductase from *A. woodii* directly using H_2 for the interconversion of CO_2 to formate (Schuchmann & Müller, 2013). Since then, several studies have demonstrated acetogenic whole-cell biocatalysis for the conversion of H_2/CO_2 to formic acid with increasing production rate thanks to proper biocatalyst selection (Schwarz et al., 2018) and process optimization in terms of pH control (Schwarz et al., 2021), dependency of conversion activity on growth phase (Schwarz et al., 2021) and medium design (Schwarz & Müller, 2020). *Thermoanaerobacter kivui* (*T. kivui*) is a remarkable candidate for formate biosynthesis since cell suspensions reached specific formate production rates of 234 $mmol\,g^{-1}_{protein}h^{-1}$ (152 $mmol\,g^{-1}_{CDW}h^{-1}$) while the volumetric formate production rate was 270 $mmol\,L^{-1}\,h^{-1}$ at 4 mg/ml (Schwarz & Müller, 2020).

TWO-STAGE PROCESSES USING PRODUCTS OF ABIOTIC CARBON DIOXIDE HYDROGENATION AS LIQUID SUBSTRATES FOR ACETOGENIC CULTURES

There is a plethora of methods to chemically convert CO_2 into value-added chemicals (Aresta & Dibenedetto, 2020; Huang et al., 2021). Within the scope of this review that focuses on bioproduction routes from CO_2 and H_2, we find it useful to remark that the products of CO_2 hydrogenation include formic acid and methanol (Wang et al., 2015). At-scale CO_2 hydrogenation processes are highlighted by recent demonstrations of methanol synthesis by Carbon Recycling International (CRI). Using locally sourced CO_2 and renewable H_2, CRI's pilot plant produces methanol at the scale of 4000 MT per year making it the world's largest CO_2 to methanol facility (https://www.carbonrecycling.is/project-goplant). Several mechanisms for formation of formic acid from CO_2 hydrogenation has been widely investigated (Gunasekar et al., 2019; Mitchell et al., 2019; Zhao et al., 2019). The main differences lie in the utilization of different kinds of catalysts, bulk/nano-metal or heterogenized molecular catalysts. Homogeneous catalytic systems are generally characterized by very high turnover frequencies but also by low catalyst concentration that ultimately results into production rate that are far from an industrial interest. On the other hand, the practical advantages of the heterogeneous catalysts, mainly the ease of separation of products from

the catalyst, are the main reasons why these systems are more and more investigated nowadays (Álvarez et al., 2017). Some examples of the most recent developed mechanisms for CO_2 hydrogenation into formic acid include the utilization of Ru(III) catalysts immobilized onto a triazine framework (Gunasekar et al., 2019) or palladium nanoparticles supported on Mo2C (Mitchell et al., 2019) or the Lewis pair (Zhao et al., 2019). A detailed description of the mechanisms of CO_2 hydrogenation to formic acid has been widely described in other review papers (Álvarez et al., 2017; Sun et al., 2021).

Methanol and formate are particularly attractive C_1 liquid intermediate feedstocks in quest for innovative bioprocessing routes of waste CO_2. In fact, they are amenable to transport and storage as well as to integration with existing fermentation infrastructure, feature improved mass transfer compared to gaseous feedstocks, and they can be microbiologically upgraded using acetogenic bacteria (Cotton et al., 2020; Kremp & Müller, 2021).

Up to date two options have emerged for developing the biotechnological valorization of methanol and formate: (i) engineering native methylotrophs or formatotrophs to improve their capacity for bioproduction, (ii) engineering synthetic methylotrophy or formatotrophy in established model species. Methylotrophic microorganisms are found in both prokaryotes and eukaryotes (Chen & Lan, 2020; Zhang et al., 2022). The eukaryotic methyltrophic yeast *Pichia pastoris* (reclassified as *Komagataella phaffii*) can metabolize methanol as its sole carbon and energy source and is one of the most widely used host for the production of recombinant protein production (Ergün et al., 2021), SCP (Shay & Wegner, 1981), and several valuable compounds such as TCA cycle intermediates (Guo et al., 2021), terpenoids, polyketides, lovastatin – an antihypertensive compound – and its precursor monacolin (Liu et al., 2018).The most well studied methylotrophs include both Gram positive (e.g. *Bacillus methanolicus*) and Gram negative (e.g. *Methylobacterium extorquens, Methylococcus capsulatus*) prokaryotic bacteria. Industrial-scale processes using methylotrophic bacteria have proven successful for providing large SCP amounts for human and animal feed. The examples from ICI (Windass et al., 1980) using *Methylophilus methylotrophus*, Hoechst/Uhde (https://doi.org/10.1021/cen-v056n020.p020) using *Methylomonas clara* and NorskHydro (Zhang et al., 2022) using *Methylomonas methanolica* illustrate that large-scale methanol-based processes are possible from the engineering point of view. Recent years have witnessed considerable progress in engineering synthetic methylotrophy in model bacteria such as in *E. coli* and *S. cerevisiae* (Keller et al., 2022; Kelso et al., 2022). Even if native formatotrophs, can grow using formic acid as a sole carbon source (Qiao et al., 2021), native formic acid assimilation pathways, including serine and reductive acetyl-CoA pathways, can be kinetically and energetically inefficient. Thus, synthetic formic acid assimilation pathways have been developed such as the formolase (Fls) pathway, the synthetic acetyl-CoA (SACA) pathway, the reconstructed THF cycle and reverse glycine cleavage pathway (rTHF-rgcv) pathway, the modified serine cycle, and the synthetic homoserine cycle (Bang et al., 2021). Nevertheless, the use of methanol and formate as microbial feedstocks comes with some challenges such as their toxicity that requires careful consideration when developing appropriate feeding strategies (e.g. methanol-limited condition) to maximize growth and product formation rates. For example, the acetogen *Butyribacterium methylotrophicumare*, which can assimilate several C_1 substrates, features one of the highest tolerance to formate and methanol, growing in up to 400 mM of formate and 1000 mM of methanol (Humphreys et al., 2022).

In acetogens, both formate and methanol enter the WLP within the methyl branch of the pathway but their entry points are different (Figure 3). In fact, formate is the first intermediate after the fixation of CO_2 and is converted into 5-methyl-tetrahydrofolate (5-methyl-THF) in four intermediate reactions that include the formation of 10-formyl-THF, 5,10-methenyl-THF and 5,10-methylene-THF intermediates. These reactions are catalysed by the 10-formyl-H4folate synthetase, 5,10-methenyl-H4folate

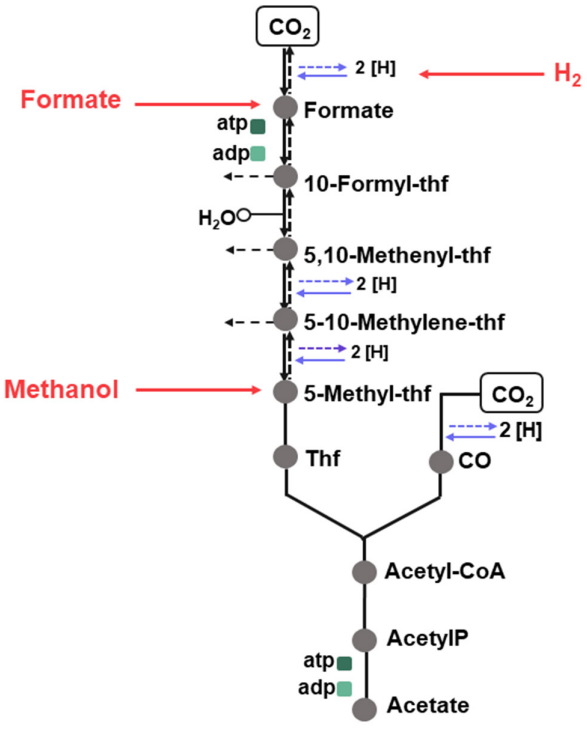

FIGURE 3 Substrate flexibility of acetogens. Methanol and formate entry points in the Wood–Ljungdahl are denoted by solid arrows. Cofactors and energy equivalents are coded, respectively, in blue and green colours.

cyclohydrolase, 5,10-methylene-H4folate dehydrogenase and the 5,10-methylene-H4folate reductase enzymes. On the contrary, methanol enters the pathway via the methanol-THF methyltransferase system to directly form 5-methyl-THF, avoiding most of the metabolic cost in the form of ATP and NAD(P)H of the methyl branch. Subsequently, the methyl group of 5-methyl-THF is transferred to a subunit of the CO dehydrogenase/acetyl-CoA synthase (CODH/ ACS) via a methyltransferase and a corrinoid iron–sulfur protein and combined to the carbonyl group of acetate that derives from a second mole of CO_2 and to Co-enzyme A (CoA) to form acetyl-CoA. Finally, acetyl-CoA is converted to acetate thanks to the action of a phosphotransacetylase and an acetate kinase (Ragsdale & Pierce, 2008; Schuchmann & Müller, 2014).

Interestingly, these feedstocks support higher energetic efficiencies of bioproduction (calculated as the fraction of the combustion energy of the substrate that is retained in the product) compared to that achieved with hydrogen (Claassens et al., 2019; Cotton et al., 2020). According to current data, growth on methanol and formate is higher than that observed under the CO_2/H_2 condition (Bache & Pfennig, 1981; Breznak et al., 1988; Dehning et al., 1989; Sharak Genthner & Bryant, 1987). Furthermore, it was interestingly noted that, when formate and methanol are simultaneously used as co-substrates, formate is useful to increase growth rate and cell density and methanol is useful to synthesize more reduced products such as butyrate and butanol (Wood et al., 2022). It is, therefore, possible to outline strategies integrating abiotic and biotic catalyses to harness their respective advantages, namely the high specificity and energetic efficiency of CO_2 hydrogenation, on one side, and the flexibility of the biological processes regarding the achievable products, on the other side. In this regard, strain optimization through genetic modifications may be necessary to steer the carbon and electron flow into the compound of interest and to prevent side-products' formation (Bourgade et al., 2021; Lee et al., 2022). Notably, the flexibility of the abiotic/biotic two-stage process benefits also of the fact that it is possible to divert methanol and formic acid intermediates to other, non-biological uses. For example, formic acid can be used either for electricity regeneration or for bioproduction of commodity chemicals (Eppinger & Huang, 2017). Such hybrid abiotic/biotic production chains open an attractive option for the conversion of CO_2 into biocommodities in a future circular carbon economy.

Renewable methanol as feedstock for acetogenic cultures

In acetogens the biochemistry and general metabolism of methanol assimilation in the methyl branch of the WLP via the methanol-THF methyltransferase system is known, is spite of still remaining uncertainties (Kremp & Müller, 2021). Depending on the organism, the electron carriers involved in methyl group oxidation and the catalysing enzymes differ, which, in the end, greatly influences the overall ATP yield. It is known that only a limited number of acetogens including *A. woodii* (Kremp et al., 2018), *M. thermoacetica* (Das et al., 2007), *S. ovata* (Stupperich & Konle, 1993; Tremblay et al., 2015), *Eubacterium limosum* (van der Meijden et al., 1984) and *Butyribacterium methylotrophicumare* (Humphreys et al., 2022) are able to grow on methanol. Not much is known about biochemical production using methanol as substrate. Recently, theoretical models of chemicals production from methanol have shown the feasibility for ethanol, lactate and acetone production in *A. woodii*, as well as for butyrate and butanol production in *E. limosum*. As aforementioned, the synthesis of valuable biochemicals from methanol could be beneficial compared to their direct production from CO_2/H_2 in terms of bioenergetics (Claassens et al., 2019; Cotton et al., 2020). When acetogens are grown on methanol, the CO_2 necessary for the carbonyl branch is supplied by running the reactions of the WLP methyl branch in the reverse direction. Since methanol is incorporated via the methanol-THF methyltransferase system, the reversal of the methyl branch results in a net gain of one ATP and two NADH equivalents. The additional NADH generated in methanol oxidation obligates NAD+ regenerating reactions for redox balance, which drives the synthesis of more reduced products such as the synthesis of butyrate by the NADH-dependent 3-hydroxybutyryl-CoA dehydrogenase (Hbd) and butyryl-CoA dehydrogenase (Bcd) enzymes. A recent study demonstrated that the yield of butyrate in *E. limosum* KIST612, grown on methanol as sole carbon and energy source, was significantly higher than that obtained under CO_2/H_2, where butyrate was produced in trace amounts (Litty & Müller, 2021). Indeed, growth on methanol led, in the stationary phase, to the formation of butyrate, with a butyrate:methanol ratio of 0.17:1 and a butyrate:acetate ratio of 0.33:1 (Litty & Müller, 2021). Further fermentation studies using *E. limosum* recapitulated higher butyrate yield on methanol than under CO_2/H_2 (Flaiz et al., 2021). The possibility for a combined chemical-biochemical production of butyrate using methanol obtained from CO_2/H_2 has been a prelude to the production of butanol, when coupled with the advent of established genetic tools (Jeong et al., 2020). Indeed, engineering *E. limosum* strains has recently translated into the production of butanol from methanol (Flaiz et al., 2021). Interestingly, the same study achieved acetone production from methanol by introducing an artificial acetone production operon from *C. acetobutylicum*. Additionally, *B. methylotrophicum* has come into the spotlight as an acetogenic chassis for the production of biotechnological compounds from methanol

since it is able to convert it into butyrate and caproate, a C_6 product, presumably through chain elongation cycles of the reverse β-oxidation pathway (Humphreys et al., 2022).

Methanol toxicity is a disadvantage in the development of methanol-based bioprocessing systems. Nonetheless, in this regard, adaptive laboratory evolution has already proved a valuable tool to enhance methanol tolerance. For instance, the evolution of a strain of the acetogen *S. ovata*, which included a modified cell wall, the possible use of osmoprotectants, and the possible modulation of chaperones' activity, led to a 5-fold increase in the growth rate on methanol (Tremblay et al., 2015). In conclusion, methanol is a promising C_1 liquid substrate for the development of biotechnologies producing multicarbon commodity chemicals.

Renewable formate as feedstock for acetogenic cultures

The C_1 compound formate is a promising substrate for producing biochemicals by formatotrophic microbes (Yishai et al., 2016). As previously outlined, formate is the first intermediate of the methyl branch of the WLP and was reported to be used as a substrate in some acetogens (Breznak & Switzer, 1986) among which *A. woodii* (Balch et al., 1977), *Eubacterium aggregans sp. nov.* (Mechichi et al., 1998), *C. scatologenes* (Küsel et al., 2000), *Acetobacterium tundrae sp. nov.* (Simankova et al., 2000) and *C. ljungdahlii* (Tanner et al., 1993), albeit at minor extent. In acetogens such as *A. woodii* and *T. kivui*, the first reaction of the WLP methyl branch is catalysed by a unique enzyme system, the hydrogen-dependent CO_2 reductase complex (HDCR), capable of oxidizing formate to H_2 and CO_2, which is superior over any chemical catalyst for formate-based H_2 production (Schuchmann & Müller, 2013). Therefore, these microorganisms are promising candidates for formate-based H_2 production (Müller, 2019). In fact, *A. woodii* reached one of the highest formate-based H_2 production performances reported so far at ambient conditions for an organism without genetic modification (Kottenhahn et al., 2018). Cell suspensions reached specific formate-dependent H_2 production rates of 30.5 mmol g_{CDW}^{-1} h^{-1} and maximum volumetric H_2 evolution rates of 79 mmol L^{-1} h^{-1}. Acetate was the major side-product that decreased the H_2 yield. Since HDCR does not require other cellular electron carriers than H_2, the catalysed reaction is independent of the cell metabolism (Schuchmann & Müller, 2013). This opened the possibility of uncoupling growth and energy conservation from the reversible reduction of CO_2 to formate with H_2 as electron donor catalysed by HDCR. Since the energy metabolism of *A. woodii* depends on a sodium ion gradient across the cytoplasmic membrane, the inhibition of the energy metabolism by adding a sodium ionophore was particularly effective, completely abolishing acetate formation. Under these conditions, yields up to 1 mol H_2 per mol formate were achieved (Kottenhahn et al., 2018). The thermophilic acetogenic bacterium *T. kivui* is an efficient biocatalyst for the oxidation of formate to H_2 and CO_2. A long-term application of *T. kivui* as a whole-cell system for formate-based hydrogen demonstrated the technical feasibility of this conversion route, which proceeded at a specific rate of 11.9 mmol g_{CDW}^{-1} h^{-1} (Schwarz et al., 2021). Under controlled reaction conditions (e.g. pH) in batch-operated stirred-tank reactors, the *T. kivui* culture achieved a H_2 production rate of 685 mmol g^{-1} h^{-1}, which is the highest reported in the literature so far for wild-type organisms. Additionally, a yield $Y_{(H2/formate)}$ as high as of 0.86 mol mol^{-1} and a hydrogen evolution rate as high as of 999 mmol $L^{-1}h^{-}1$ were observed using 4 mg/ml cell protein (Burger et al., 2022). This rate is higher than the highest rate described for the wild-type acetogenic bacterium *A. woodii* (Kottenhahn et al., 2018), and among the highest rates reported for wild-type H_2-producing microorganisms (Lim et al., 2012).

In order to pursue formate-based bioproduction systems, several aspects of formate metabolism are worth of attention like formate initial metabolism including formate transport mechanisms (Moon et al., 2021). For instance, an adaptive laboratory evolution approach, which enhanced the hydrogen production of the hyperthermophilic archaeon *Thermococcus onnurineus*, pinpointed a mutated formate transporter as a critical adaptive passage (Jung et al., 2017). Another aspect of formate metabolism that is attracting increasing attention is the existence of pathways enabling pyruvate synthesis from formate. Since pyruvate is a central intermediate in biosynthetic pathways, pyruvate production from formate would sustain novel strategies for formate fixation in biotechnologically relevant compounds (Müller, 2022). In this regard, it is interesting to note that the *A. woodii* genome encompasses three putative genes encoding pyruvate:formate lyases, which have recently been found to condense acetyl-CoA and formate into pyruvate in the model bacterium *E. coli* (Kirst et al., 2022; Zelcbuch et al., 2016). In summary, CO_2-based formate can become an ideal intermediate between the hydrogenation of CO_2 and bioprocessing technologies in the energetic and chemical sectors.

CARBON AND ENERGY BALANCES FOR SELECTED FIRST AND SECOND STAGE PROCESSES

In order to assess the overall carbon and energy yields from reactants to products, C- and energy-balances were calculated for selected cases based on the information available in the original publications.

Regrettably, authors do not always present closed C-molar balances or C-molar yields, as well as H- or energy-balances of the respective processes. Hence, for assembling carbon and energy balances out of published data, assumptions need to be taken (e.g. C-molar biomass weight, stoichiometric H_2/CO_2 ratio).

In the present publication, by way of example the carbon and energy balance regarding the conversion from reactant to product was calculated for the following processes:

First stage:

(i) formic acid production with *A. woodii* from H_2/CO_2 (Schwarz et al., 2021),
(ii) acetate production from H_2/CO_2 with *C. ljungdahlii* (Hermann et al., 2020),
(iii) ethanol production from H_2/CO_2 with *C. autoethanogenum* (Heffernan et al., 2020).

Second stage:

(i) PHB production from acetate with *R. eutropha H16* (Al Rowaihi et al., 2018),
(ii) PHB production from acetate with *C. basilensis* (Cestellos-Blanco et al., 2021),
(iii) PHB production from ethanol with *E. coli* Q3094 (Sun et al., 2020).

First stage

Yields (molar or C-molar, depending on what was reported) from CO_2 to product and biomass have been extracted from the specified paper to elaborate the C-balance. In order to determine the respective stoichiometric amount of required H_2, an H-balance was set up and H_2O generation was assumed to close this balance (to ±0.1%). For elemental balancing of reactions, we used the protonated forms of formate and acetate (so formic acid and acetic acid). The low heating value (LHV) for H_2 was used for the energy balance while the LHVs of all other components were calculated based on the formulas reported in ref. (Hosokai et al., 2016). For formate and acetate, we used the deprotonated form for LHV calculation. Biomass was assumed to have the following simplified elemental composition for all cases: $CH_2O_{0.5}$, the biomass C-molar weight, if not reported in the original publications, was assumed equal to 25 g/C-mol. We calculated the two-stage energy and carbon yields by multiplying the yields of the two respective stages.

Second stage

Balances were not closed, since not relevant for calculating carbon or energy yields for the product of interest (no fed H_2, no other carbon source but acetate) and information on other educts or by-products were not reported. For PHB production, if not reported differently in the original publications, the extracted biomass yields from literature were assumed to be without PHB content and on a C-molar basis and the PHB yields were interpreted on a molar basis. The following elemental composition was used $C_4H_6O_2$. LHV calculation was done in the same way as for the first stage processes.

The energy and carbon yields when we considered acetate and formate formation from CO_2/H_2 are neatly higher than in the case of ethanol (Table 3, Table S2). As representative example of an integrated two-stage process, we then sought to evaluate the carbon and energy yield of two-stage processes aimed at PHB production using either acetate or ethanol as substrate. The energy and carbon yield of the gas fermentation processes in the first stage are superior to the energy and carbon yields pertaining the PHB production, independently on whether acetate or ethanol acts as carbon source (Table 2, Table S1). Nonetheless, the overall energy and carbon yields of the two stages are still noticeable, especially in the light of the fact that the two-stage process actually fixes CO_2 using H_2 as reducing agent. Moreover, the CO_2 generated in the PHB-producing stage could be recycled and used in the first stage. In the same vein, the biomass could be used to supply the yeast extract used in the media of both stages following extraction of the target product.

CONCLUDING REMARKS

Carbon dioxide recycling is a compelling necessity and microbial carbon dioxide fixation in value-added compounds is a valuable opportunity. Fermentation of CO_2 gas streams using acetogenic bacteria is consolidating as a key biotechnology to move toward a cyclic carbon economy. All microorganisms that capture CO_2 require an energy source. H_2 is considered as the preferable electron donor source for an efficient CO_2 fixation via the WLP, since it affords no loss of carbon in CO_2 dissipated (Hermann et al., 2020; Valgepea et al., 2018). Most of H_2-based CO_2 fermentations are single stage processes. Even though the number of added-value compounds achievable by H_2-based CO_2 fermentation is on the rise (Lauer et al., 2022; Mook et al., 2022; Weitz et al., 2021) using metabolically engineered acetogens (Bourgade et al., 2021; Lee et al., 2022; Song et al., 2022), high-profile demonstrations of process scalability is restricted to acetate and ethanol (Fackler et al., 2021). A possible strategy to broaden the product spectrum of CO_2 gas fermentation consists of considering the gas fermentation mediated by acetogens in the context of two-stage processes. Indeed, the two-stage approaches discussed in our review potentially opens a multitude of biotechnological options to convert waste

TABLE 3 Carbon and energy balances corresponding to acetate, formate, and ethanol production with a pure acetogenic culture grown on a gas mixture consisting of CO_2 and H_2.

Formate (Schwarz et al., 2021)	**Carbon dioxide**	**Hydrogen**	**-->**	**Biomass**	**Formate**	**Water**		**Energy balance [kJ]**	**Energy yield formate [%]**
Molar formula	CO_2	H_2	-->	$CH_2O_{0.5}$	CO_2H_2	H_2O			
Reaction stoichiometry [moles]	1	1	-->	0	1.00	0			
Energy content [kJ/mol]	0	242.0	-->	529.7	181.2	0			
Energy balance [kJ]	0	242.0	-->	0	181.2	0		60.83	74.87
Reaction stoichiometry [C-moles]	1		-->		1				
Acetate (Hermann et al., 2020)	**Carbon dioxide**	**Hydrogen**	**-->**	**Biomass**	**Acetate**	**Ethanol**	**Water**	**Energy balance [kJ]**	**Energy yield Acetate [%]**
Molar formula	CO_2	H_2	-->	$CH_2O_{0.5}$	$C_2O_2H_4$	C_2OH_6	H_2O		
Reaction stoichiometry [moles]	1	2.035	-->	0.031	0.4650	0.0095	1.045		
Energy content [kJ/mol]	0	242.0	-->	529.7	802.4	1233	0		
Energy balance [kJ]	0	492.5		16.42	373.1	11.72	0	91.27	75.76
Reaction stoichiometry [C-moles]	1		-->	0.031	0.930	0.0190			
Ethanol (Heffernan et al., 2020)	**Carbon dioxide**	**Hydrogen**	**-->**	**Biomass**	**Ethanol**	**Acetate**	**Water**	**Energy balance [kJ]**	**Energy yield Ethanol [%]**
Molar formula	CO_2	H_2	-->	$CH_2O_{0.5}$	C_2OH_6	$C_2O_2H_4$	H_2O		
Reaction stoichiometry [moles]	1	2.545	-->	0.04	0.27	0.209	1.271		
Energy content [kJ/mol]	0	242.0	-->	529.70	1233.40	802.4	0		
Energy balance [kJ]	0	616.0	-->	20.66	336.10	167.3	0	91.90	54.57
Reaction stoichiometry [C-moles]	1		-->	0.039	0.545	0.417			

Note: In order to compute carbon and energy balances we collected the available information on formic acid production with *A. woodii* (Schwarz et al., 2021), acetate production with *C. ljungdahlii* (Hermann et al., 2020) and ethanol with *C. autoethanogenum* (Heffernan et al., 2020) from CO_2/H_2 gas fermentation. Details on the computation are provided in the main text and Table S2.

CO_2 to value-added chemicals by means of clean H_2. Compared to single-stage bioprocessing systems, the unique advantages of two-stage processes include enhancement in process titre/rate/yield metrics, the widening of the achievable multi-carbon products portfolio, and increased flexibility in infrastructural implementation and operation. Furthermore, compared to simultaneous co-culture systems (Diender et al., 2021; Du et al., 2020) or undefined mixed culture fermentation (Arslan et al., 2012; Calvo et al., 2021) that are capturing attention, chaining different (bio-)technological processes in cascade allows for individual unit optimization, overall biomanufacturing modularity and target product selectivity. Another advantage is the opportunity to implement circularity concepts within the high-level integrated design of the double-stage process itself. For instance, recycling of the CO_2 tail gas likely generated from the secondary fermentation to the primary one could be an example yielding a significant overall rate of CO_2 fixation. In the same vein could be the integration of nutrients between the primary and secondary fermentation. When acetate is the intermediate coupling the first and second stage of the process, the secondary fermentation consumes acetic acid, and the pH of the permeate of the secondary fermentation is nominally higher than the pH of the acetate-containing broth. The acetate-depleted broth can be returned to the primary fermentation. In this way, it can contribute to reduce the cost of pH control relative to the first stage of the process where pH is controlled only by direct addition of base to the bioreactor medium.

Throughout the review we pinpointed an ample range of products that are technically attainable by reframing a CO_2-based gas fermentation process within a two-stage context with the aim of highlighting some avenues available for fruitful exploitation of the current technology. There are many fields of application where the products achievable by the two-stage processes here discussed can compete with established chemicals for existing markets or can create entirely new ones. This variety of possible uses means that products' market prices may vary considerably, ranging for instance from <$10/kg for PHB to $73/kg for phloroglucinol. Products' market value needs careful consideration in order to evaluate if the processes under development can gain economies of scale. The issue of transferability of the outlined breakthroughs to practice still needs to be addressed at multiple levels. Although the envisaged two-stage processes are primed to favourable sustainability metrics, it is worthwhile to underscore the gaps still remaining in quantitative and critical knowledge of the individual stages and, particularly, of the integration thereof. Along with biocatalyst development, an industrial biotechnology process includes the assessment of biocatalysts' performance in scaled-up environments. Underevaluating the design, development and operation of the technological equipment can make the potential of otherwise excellent biocatalysts unrealized. Similarly, scarce analysis of the life-cycle impact and of the economic return of the process can prevent the translation of laboratory-scale biocatalysts into real bioprocess of a sustainable bioeconomy. With these challenges ahead, it is undoubtful that the outlined processes hold real transformative potential to realize innovative and sustainable value chains.

AUTHOR CONTRIBUTIONS

Luca Ricci: Data curation (supporting); investigation (equal); writing – original draft (supporting); writing – review and editing (supporting). **Arne Seifert:** Data curation (supporting); formal analysis (equal); writing – original draft (supporting). **Sebastien Bernacchi:** Data curation (supporting); formal analysis (equal); writing – original draft (supporting). **Debora Fino:** Funding acquisition (supporting); writing – review and editing (supporting). **Candido Fabrizio Pirri:** Funding acquisition (supporting); writing – review and editing (supporting). **Angela Re:** Conceptualization (lead); data curation (lead); formal analysis (equal); funding acquisition (supporting); investigation (lead); methodology (equal); visualization (lead); writing – original draft (lead); writing – review & editing (lead).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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