Conversion of Carbon Monoxide to Chemicals Using Microbial Consortia

One-Carbon Feedstocks for Sustainable Bioproduction

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https://doi.org/10.1007/10_2021_180

Adv Biochem Eng Biotechnol (2022) 180: 373–408
https://doi.org/10.1007/10_2021_180

Published online: 23 November 2021

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Abstract Syngas, a gaseous mixture of CO, H_2 and CO_2, can be produced by gasification of carbon-containing materials, including organic waste materials or lignocellulosic biomass. The conversion of bio-based syngas to chemicals is foreseen as an important process in circular bioeconomy. Carbon monoxide is also produced as a waste gas in many industrial sectors (e.g., chemical, energy, steel). Often, the purity level of bio-based syngas and waste gases is low and/or the ratios of syngas components are not adequate for chemical conversion (e.g., by

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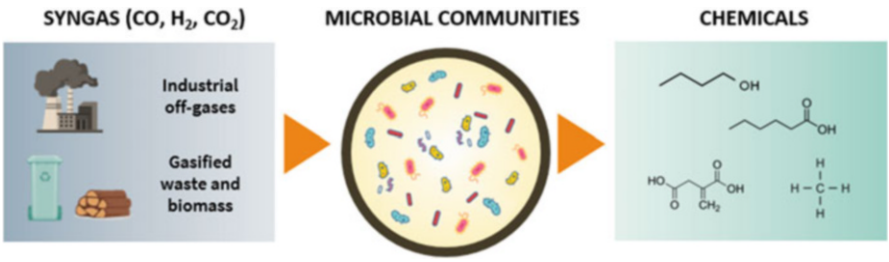
Fischer-Tropsch). Microbes are robust catalysts to transform impure syngas into a broad spectrum of products. Fermentation of CO-rich waste gases to ethanol has reached commercial scale (by axenic cultures of *Clostridium* species), but production of other chemical building blocks is underexplored. Currently, genetic engineering of carboxydotrophic acetogens is applied to increase the portfolio of products from syngas/CO, but the limited energy metabolism of these microbes limits product yields and applications (for example, only products requiring low levels of ATP for synthesis can be produced). An alternative approach is to explore microbial consortia, including open mixed cultures and synthetic co-cultures, to create a metabolic network based on CO conversion that can yield products such as medium-chain carboxylic acids, higher alcohols and other added-value chemicals.

Graphical Abstract



Keywords Acetogens, C1 feedstocks, Cross-feeding, Gas fermentation, Microbial consortia, Microbial interactions, Syngas

1 Introduction

1.1 Syngas Fermentation for a Circular Economy

As the worldwide population grows and the consumption of fossil resources increases, there is the need to develop new technologies to produce commodity chemicals from renewable resources. By 2050, chemicals may no longer be synthesised from fossil fuels, according to targets established after the Paris agreement and the European Green Deal [1, 2]. Lignocellulosic biomass and wastes (agricultural, industrial and municipal) have been identified as priority feedstocks for a bio-based industry [3, 4]. These are inedible materials, and their use does not compete with human or animal nutrition or with the utilisation of arable land, therefore circumventing ethical concerns. Wastes in particular are heavily underutilised materials, especially in developing countries [5]. The conversion of biomass and wastes through hydrolysis-fermentation is very attractive, but bottlenecks of the process are the low biodegradability of lignin (which represents 10–25% of plant

biomass) and the costly pre-treatment steps [6, 7]. An alternative that gets increasing attention is the gasification of biomass and wastes followed by the chemical or biological conversion of the generated synthesis gas (also known as syngas) [8, 9]. Syngas is a gas mixture of mainly carbon monoxide (CO), hydrogen (H_2) and carbon dioxide (CO_2) that can be generated from solid carbonaceous feedstocks (e.g., coal, lignocellulosic biomass) and carbon-containing wastes (e.g., agricultural waste). Chemical conversion of syngas by, e.g., Fischer-Tropsch (FT) process is a mature technology used for the conversion of mainly coal-generated syngas into hydrocarbons, alcohols and organic acids [10]. FT processes use metal catalysts under high temperature and pressures and require high H_2:CO molar ratios. Chemical catalysts are highly sensitive to syngas impurities such as ammonia [11], sulphur species [12], alkali ions [13] or water [11], which makes them less suitable for the treatment of biomass/waste-generated syngas. Gas clean-up treatments can reduce the concentration of most impurities, but complete removal is hindered by the cost of these technologies and the inherent variability of the feedstock [14]. Biological conversion of syngas involves its fermentation by microorganisms, which are in general more resistant to impurities in the gas and, in addition, do not require a fix H_2:CO molar ratio [8, 15–17]. The biological route operates under mild temperature and pressure conditions, and overall has higher mass and energy conversion efficiencies compared to chemical catalysis [9, 18]. Furthermore, microbial processes result in higher product selectivity with the formation of fewer by-products. Syngas fermentation technology can also be applied for the treatment of CO-containing waste gases from heavy industry such as steelmaking. Often, CO-rich off-gases gases from steel mills are burned leading to CO_2 emissions; in 2019, on average 1.83 tonnes of CO_2 were emitted per every ton of steel produced [19], contributing to approximately 8% of global emissions. This is a serious environmental problem with impact on climate change. Other opportunities are emerging to use gas fermentation technology associated to CO_2 capture technology. For example, the production of CO by electrochemical reduction of CO_2 has been proved feasible and with high Faraday efficiencies (>80%) [20–22].

1.2 Microbes Using Carbon Monoxide for Growth

Microbes have exploited CO as sustenance for much of their evolutionary history. Proof of that are the different ways in which CO may be involved in microbial metabolism, which makes it necessary to define some terms. Microorganisms that can use CO as carbon and energy source are denominated *carboxydotrophs*, to be distinguished from *carboxydovores*, which may use electrons from CO but require organic carbon for growth [23]. At the same time, CO metabolism can take two forms: respiratory and fermentative [24]. The former relies either on O_2 (aerobic) or other external electron acceptors (anaerobic). In this review, the focus is on the latter: fermentative CO metabolism, which is, by definition, anaerobic. Microorganisms that ferment CO are carboxydotrophic. Therefore, the term *carboxydotroph* is used

in this text to refer to the ability to use CO anaerobically but, in another context, it may refer to both aerobic and anaerobic microorganisms.

The fermentation of CO/syngas is carried out by acetogens, a specialised group of anaerobic bacteria able to use CO and H_2/CO_2 as sole carbon and energy sources via the reductive acetyl-CoA pathway, also known as the Wood-Ljungdahl pathway (WLP) [8]. Acetogenesis is not a phylogenetic trait; it is widely represented in at least 23 bacterial genera [25, 26]. Most known acetogens belong to the genera *Clostridium* and *Acetobacterium*, within the Clostridia class. The WLP results in acetyl-CoA as end-product of CO and H_2/CO_2 fermentation. Since autotrophy via the WLP is energetically limited, most acetyl-CoA is directed towards acetate production to generate ATP. Thus, the majority of acetogens produce acetic acid as sole metabolic end-product. Some microorganisms can derive other chemicals from acetyl-CoA as intermediate. For example, *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, *Clostridium ragsdalei* and *Alkalibaculum bacchi* are able to produce ethanol; *C. autoethanogenum*, *C. ljungdahlii* and *C. ragsdalei* can also produce 2,3-butanediol (2,3-BDO); *Eubacterium limosum* and *Butyribacterium methylotrophicum* are able to produce butyrate; and *Clostridium carboxidivorans* can produce butyrate, butanol, caproate and hexanol [27].

The key enzyme of CO oxidation to CO_2, carbon monoxide dehydrogenase, is present in other anaerobic microorganisms that harbour variations of the WLP. Besides acetogenic bacteria, CO can be used as electron donor and/or carbon source by some methanogenic archaea and sulphate-reducing bacteria [28]. However, compared to acetogens, methanogens and sulphate-reducing bacteria are more sensitive to elevated levels of CO.

Syngas fermentation processes can be implemented with pure cultures of acetogens or with microbial communities. This chapter focuses on the latter: undefined and defined consortia of microorganisms that convert syngas to biochemicals of interest. For an overview of monoculture-based processes, we refer to recent reviews [17, 29, 30].

1.3 The Microbial Consortia Approach for Syngas Fermentation

The fermentation of syngas has been most studied and implemented in industry using pure cultures of acetogens [8, 16]. From a process perspective, monocultures are easy to control and predict, since optimal conditions for growth are well-defined. Process conditions can be tuned to target a product of interest with high selectivity and yields. As an example, the highest ethanol concentration (48 g L^{-1}) and volumetric productivity (369 g L^{-1} day^{-1}) from syngas were achieved with pure cultures of *C. ljungdahlii* [31, 32]. However, a major limitation of the use of monocultures for syngas conversion is the energetic constraint in the formation of products other than acetate and ethanol [33]. Genetic and metabolic engineering

of Clostridia strains has advanced remarkably in the last decade, paving the way towards the expression of heterologous products and enhanced yields [34, 35]. For instance, industrially relevant titres have recently been achieved for acetone, iso-propanol and 2,3-BDO [36]. Yet, important hurdles remain to be addressed in genetic engineering of Clostridia to further expand the product portfolio of syngas fermentation, such as low DNA transformation efficiencies, insufficient high-throughput recombineering tools and, in general, the need for a better understanding of acetogenic platforms at the molecular level [37]. Another disadvantage of monoculture-based strategies is the lack of robustness against process fluctuations. This is of particular relevance in the case of syngas fermentation, since the gas composition varies depending on the source or gasification method [38]. Mixed cultures are less affected than monocultures of carboxydotrophs by changes in the syngas composition [39], and are also expected to be more robust against syngas impurities (e.g., nitrogen oxides, tars), which have been shown to inhibit cell growth or interfere with product distribution in monocultures of acetogens [40].

Microbial consortia are emerging as a promising strategy aimed at overcoming the limitations of monocultures and taking syngas fermentation a step forward [30, 41, 42]. In nature, microbes rarely thrive alone; instead, cooperation and communication with other microorganisms are extremely important for survival [43, 44]. Communities can perform complicated functions that individual populations cannot, for instance the production of energy-demanding products. Compared to monocultures, microbial consortia can convert much more complex substrates and have better robustness, both because of a highly diverse community structure and a capacity to evolve. The capabilities of microbial consortia have long been exploited in bioremediation, wastewater treatment and the production of fermented foods [45]. In the last decade, advances in -omics approaches and a greater understanding of microbial interactions have driven forward the fields of microbiome engineering and synthetic ecology, aimed at unlocking the full potential of microbial communities for biotechnological applications [46–50].

The use of microbial consortia in syngas fermentation has specific advantages. For one thing, communities composed of multiple carboxydotrophic microbes with different CO tolerance can handle syngas streams with variable composition. This functional redundancy may enhance gas consumption and mitigate the detrimental effect of syngas contaminants on individual populations. Moreover, provided that carboxydotrophic populations keep CO levels low, CO-sensitive microbes can thrive in an environment that would otherwise be hostile. In addition, the co-culture capabilities can be extended by syntrophic interactions between species in the consortia, such as cross-feeding of intermediates (e.g., acetate, ethanol) or exchange of essential nutrients (e.g., amino acids, vitamins). An example of this is the co-culture of *Citrobacter amalonaticus* Y19 and the acetogen *Sporomusa ovata*. The latter has been reported to produce acetate from CO, but at rather low rates [51, 52]. On the other hand, *C. amalonaticus* Y19 is unable to use autotrophic substrates but it can oxidise CO to H_2 and CO_2 [53], which may be used as substrates by the acetogen. A study found that co-cultures of *S. ovata* and *C. amalonaticus* Y19 produced almost double the amount of acetate than monocultures of *S. ovata*, from

the same amount of CO [52]. In addition, growth of both microorganisms and CO consumption was higher in the co-culture than in monocultures. This example is just one of many that demonstrate the relevance of mutualistic interactions in microbial consortia [42, 54]. There are, on the other hand, potential downsides when using consortia of microorganisms compared to monocultures. Some examples are the occurrence of competing or inhibiting pathways, the generation of side products that reduce product selectivity or incompatible cultivation conditions (i.e., pH, temperature, etc.) between species in the community. These issues can be tackled through rational microbial consortia engineering, multi-species metabolic modelling and bioreactor/bioprocess design [47, 55, 56].

Two types of microbial consortia can be distinguished: open mixed cultures (also referred to as 'open cultures', 'mixed cultures' or 'microbiomes') and synthetic co-cultures (Fig. 1). The former consist of self-assembled, highly diverse microbial communities naturally occurring in defined habitats, in which the populations are mostly unspecified. Synthetic co-cultures are consortia of specified microbial strains that engage in interaction under aseptic and controlled conditions. Most synthetic co-cultures reported in literature are composed of two or three microbial species, with a few including up to five [57].

The following sections summarise the main developments regarding the implementation of open mixed cultures (Sect. 2) and synthetic co-cultures (Sect. 3) in syngas fermentation processes.

2 CO Conversion by Open Mixed Cultures

2.1 Anaerobic Sludges as Biocatalysts for Syngas Fermentation

The main components of syngas (CO, H_2 and CO_2) can sustain anaerobic growth of a number of microbial groups: acetogens and hydrogenogenic bacteria, carboxydotrophic and hydrogenotrophic methanogens and sulphate-reducing microorganisms [24]. In turn, the products of CO/syngas fermentation (mainly H_2, CO_2, acetate and ethanol) can support growth of acetoclastic methanogens, chain-elongating bacteria, ethanol oxidisers, syntrophic acetate-oxidising bacteria and propionibacteria, among others [16, 58]. The range of final products that can be obtained via syngas fermentation by open mixed cultures (in the absence of external electron acceptors) therefore includes short- and medium-chain carboxylic acids, simple and higher alcohols and methane. When sulphate is available and sulphate-reducing microorganisms are present, sulphide is also produced. An overview of some of the works on syngas/CO conversion by mixed cultures is shown in Table 1.

Open mixed cultures for syngas fermentation are based on inocula from anaerobic natural or engineered environments that harbour a high microbial diversity. Typical inocula include anaerobic digester sludges [66, 70–76], wastewater treatment

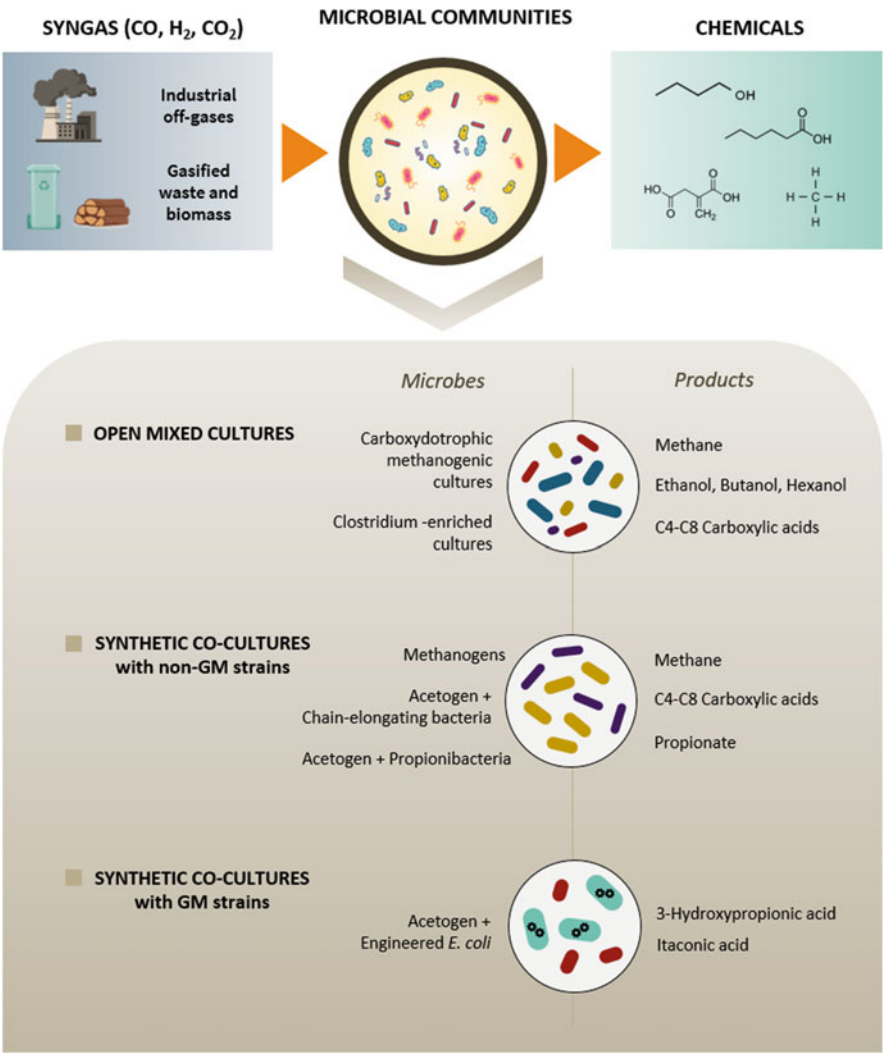


Fig. 1 Overview of the types of microbial communities that can be applied in syngas fermentation to produce biochemicals. Syngas (CO, H_2 and CO_2) can be obtained via the gasification of organic wastes or lignocellulosic residues. Off-gases from the steel and iron industries are also sources of CO-rich gas. Open mixed cultures (from, e.g., sludges) enriched in carboxydotrophic, methanogenic and/or Clostridia species can be used to produce methane, alcohols or medium-chain carboxylic acids from syngas. Alternatively, synthetic co-cultures can be used, composed of non-engineered microorganisms alone or in combination with genetically engineered platform organisms such as *Escherichia coli*. The latter option allows to expand the range of products that can be obtained from syngas to compounds of added-value, such as 3-hydroxypropionic acid or itaconic acid. Acronym: GM, genetically modified

Table 1 Overview of syngas fermentation processes using open mixed cultures

Cultivation system	*T* (°C)	pH	Enriched microbial taxa (relative abundance)	Syngas composition (% v/v)	Product titre/ productivity	Reference
TBR (lab-scale)	60	7	Biofilm: *Methanothermobacter* (30%), *Therminicola* (16%), *Coprothermobacter* (23%) Liquid: undefined (30%), *Therminicola* (23%), *Methanothermobacter* (15%)	$CO/H_2/CO_2/N_2$ (20:45:25:10)	Methane 8.49 mmol L_{bed}^{-1} h^{-1}	Asimakopoulos et al. [59]
TBR (7.5L)	60	7	Derived from Asimakopoulos et al. [59]	$CO/H_2/CO_2/N_2$ (20:45:25:10)	Methane 17.6 mmol L_{bed}^{-1} h^{-1}	Asimakopoulos et al. [60, 61]
TBR coupled to FBG	60	7	Derived from Asimakopoulos et al. [59]	$CO/H_2/CO2/N_2$ (11:35:44:10)	Methane 14.4 mmol L_{bed}-^{1} h^{-1}	Asimakopoulos et al. [60, 61]
Floating MBR	55	8.2	N.A.	$CO/H_2/CO_2$ (55: 20:10)	Methane 1.43 mmol L-^{1} h^{-1}	Chandolias et al. [62]
MOBB	35–37	5.8–6.7	N.A.	$CO/H_2/CO_2$ (60: 30:10)	Methane 3.04 mmol L^{-1} h^{-1}	[63]
HfMBR	55	6.5	Biofilm: *Thermoanaerobacterium* (92.8%)	CO/H_2 (40:60)	Acetate 24.6 g L^{-1} 16.4 g L^{-1} day^{-1}	Shen et al. [64]
HfMBR	35	6	Biofilm: *Clostridium* (41.6%), undefined (42%)	CO/H_2 (40:60)	Butyrate 1.4 g L^{-1} Caproate 0.88 g L^{-1} Caprylate 0.53 g L^{-1}	Shen et al. [64]
Column reactor filled with porous pad	35	7 to 5.8–6.5[a]	*Acinetobacter*, *Alcaligenes*, *Rhodobacter*, *Methanobacterium*, *Methanoseaeta*	CO/N_2 (60:40)[b]	Caproate 0.22 g L^{-1} Heptanoate 0.21 g L^{-1} Caprylate 0.15 g L^{-1}	He et al. [65]
HfMBR	35	4.5	Biofilm: *Clostridium* (86.3%)	CO/H_2 (60:40)	Ethanol 16.9 g L^{-1}	Wang et al. [66–68]
CSTR with cell-recycle	37	7	*A. bacchi* (56%), *A. propionicum* (34%), *Clostridium sp.* (10%)	$CO/H_2/N_2$ (28: 60:12)	Ethanol 8 g L^{-1}; Propanol 6 g L^{-1} Butanol 1 g L^{-1}	Liu et al. [69]

STR	37	7–4.3[a]	N.A.	$CO/H_2/CO_2/N_2$ (32:32:8:28)	Butyrate 2.17 g L^{-1} Butanol 0.43 g L^{-1}	Ganigué et al. [70]
STR	37	6–4.8[a]	*C. ljungdahlii*, *C. carboxidivorans*, *C. kluyveri*	$CO/H_2/CO_2/N_2$ (32:32:8:28)	Butanol 1.1 g L^{-1} Hexanol 0.6 g L^{-1}	Ganigué et al. [71]
STR	33	4.9	N.A.	CO (100)	Ethanol 11.1 g L^{-1} Butanol 1.8 g L^{-1} Hexanol 1.5 g L^{-1}	Chakraborty et al. [72]

TBR trickle-bed reactor, *FBG* fluidised bed gasifier, *MBR* membrane bioreactor, *MOBB* multi-orifice baffled bioreactor, *HfMBR* hollow-fibre membrane bioreactor, *STR* stirred-tank reactor (batch), *N.A.* not available data

[a]The pH was initially set and not controlled afterwards

[b]Percentage at the end of the process, with an inflow syngas composition of 60 kPa CO and N_2 as make-up gas, assuming a total pressure of 101 kPa

granules [76, 77] and faeces of herbivores [78–82]. Sludges from anaerobic digesters employed in traditional wastewater treatment processes have been proposed as most suitable syngas biocatalysts [73, 76, 77]. These cultures have a high adaptation capacity, essential to treat a vast range of organic and inorganic substrates. Most importantly for syngas fermentation, the ability to oxidise CO seems to be a ubiquitous property across anaerobic sludges [76].

An acclimation period is generally required to obtain a microbiome capable of efficiently converting CO. The inoculum largely determines the duration of this acclimation period, which can be as short as a few days [76, 77, 79, 81] or weeks [67, 71, 76], and in some cases lasts several months [65, 73, 75]. Because different microbes have different optimal conditions for growth, the outcome of the process depends not only on the inoculum source but also on the environmental conditions applied to enrich and maintain the culture. Acetogenic bacteria are more tolerant to CO than other microorganisms present in anaerobic sludges, therefore dominating enriched cultures exposed to moderate or high levels of CO [64, 66, 67, 75, 83–85]. For example, during the CO-enrichment process of an anaerobic sludge, the relative abundance of members from the Clostridiales order, which includes many acetogenic species, increased from 5% in the inoculum to 66–95% in enriched cultures (pCO = 20–61 kPa) [75]. Similarly, a recent study showed that mixed cultures exposed to high pCO (96 kPa) were dominated by members of the Firmicutes phylum, to which many acetogens belong, while low pCO (35 kPa) shifted the community towards Proteobacteria, a phylum that includes hydrogenogenic carboxydotrophs [83]. Methanogens, on the other hand, are generally inhibited at moderate CO pressures starting from 30 to 80 kPa [73, 75, 77]. In a recent study, Duan et al. [83] revealed the crucial role of under-characterised taxa in CO-enriched communities. Authors identified novel bacterial genera and species which may participate in CO oxidation to end-products and maintain fundamental metabolism (e.g., citric cycle, amino acid biosynthesis), extending the functional redundancy of the communities and overall increasing their stability. Besides CO, the presence of other gases in the mixture has an impact on the performance of syngas-converting communities. With few exceptions, pure cultures of carboxydotrophs can rarely consume H_2 and CO simultaneously, since almost all hydrogenases are inhibited by CO [86–91]. In contrast, mixed cultures can metabolise H_2 along with CO, since H_2 can be used by hydrogenotrophic microorganisms that might be present in the community [39]. Overall, the addition of CO_2 and H_2 has been shown to increase the microbial diversity of CO-enriched cultures and promote a higher acetate/ethanol ratio [39, 75].

Temperature and pH are two operational parameters with a major influence on the evolution of CO enrichments. Grimalt-Alemany et al. [92] showed that mesophilic syngas-enrichments (37°C) are characterised by a higher microbial diversity and a more intricate metabolic network compared to thermophilic syngas-enrichments (60°C). Another finding of that study was that the maximum specific growth rates of microbes were significantly higher (twofold) in thermophilic conditions. Similar

findings were reported by Alves et al. [73], who observed a rapid decrease in microbial diversity in long-term CO/syngas enrichments of anaerobic sludges at 55°C. The pH is perhaps the most crucial parameter determining the structure and product composition of syngas-converting communities. Several authors have emphasised its critical effect on the regulation between acetogenesis and solventogenesis, requiring a tight control of acidic conditions [71, 72, 84, 85], while, for methanogenesis, neutral or slightly alkaline conditions are required [84, 85].

Finally, the addition of medium supplements (e.g., yeast extract, reducing agents) and inhibitors of specific types of metabolism is also common practice to alter the structure of microbial communities and stimulate the production of target products [72, 92, 93]. In this regard, the addition of methanogenic inhibitors is an extended practice in syngas fermentation processes using open mixed cultures to supress methane production. Inocula from anaerobic sludges are likely to harbour an active methanogenic population. This can be a hurdle when products other than methane are targeted. Three approaches are commonly used to inhibit methane production by open mixed cultures used in bioprocesses, namely (1) operation under (mildly) acidic conditions [94, 95], (2) heat-shock treatment of the inoculum [95, 96], and (3) the addition of methanogenic inhibitors such as 2-bromoethanesulphonate (2-BES) [70, 71, 95, 97, 98]. The latter, in concentrations ranging 10–50 mM, has proven very efficient and is therefore a popular choice; however, it can certainly contribute to increasing process costs at industrial scale since periodical addition is necessary in continuous operation. Moreover, 2-BES can lose efficacy during long-term operation [65] and can be metabolised by dehalogenating and sulphate-reducing bacteria present in microbial communities [95, 99]. Eventually, moderate to high concentrations of CO should inhibit most methanogenic activity and avoid the addition of specific inhibitors.

One of the big advantages of using open cultures in bioprocesses is that these do not require operation under aseptic conditions. In addition, long-term reproducibility of mixed cultures can be ensured by using suitable cryopreservation methods [100]. On the other hand, these systems are highly dependent on microbial interactions, which are very difficult to predict and, to a great extent, unknown. Other drawbacks are the long times required to achieve steady-state conditions and the challenging product recovery due to the presence of many by-products at low concentrations. Large-scale continuous processes using open mixed cultures are well-established in industry (e.g., in wastewater treatment and food fermentation), but not yet applied for syngas fermentation. Decades of research have shed light on the structure of anaerobic sludges, their governing microbial interactions and the influence of operational parameters, although mainly in the context of conversion of organic compounds [68, 101–103]. However, developments in the last decade are driving forward the syngas fermentation platform for mixed cultures at a rapid pace. The next sections relate these advances, centred on the production of methane, carboxylic acids and alcohols.

2.2 Syngas Biomethanation

Conversion of CO-rich waste gases by open mixed cultures is a popular method to produce methane [84, 85, 104]. Traditional wastewater treatment processes rely on microbial communities that perform methanogenesis as ultimate step of anaerobic digestion. These cultures are suited to produce methane from syngas, provided they have or acquire a sufficient carboxydotrophic potential [77]. Biomethanation of syngas presents several advantages over its analogous catalytic process [84, 85, 105]. Microbes are less sensitive than metal catalysts to impurities and to the ratio C/H in syngas. Biocatalysts are cheap, self-replicating, and can yield high methane contents in a single step. Using microbes, higher methane selectivity can be obtained, in contrast to the use of metal catalysts that result in the production of higher hydrocarbons as by-products. However, production rates of biomethanation are lower compared to the chemical process. Recent years have witnessed increased efforts to improve the efficiency of syngas biomethanation; these include the development of novel reactor configurations, insights on the impact of operational parameters and improved knowledge on the microbial community structure and interactions [84, 85].

Methanogenesis from CO can take place via three routes: (1) direct conversion by carboxydotrophic methanogens, (2) via acetate as intermediate by acetoclastic methanogens, and (3) via H_2/CO_2 as intermediates by hydrogenotrophic methanogens. Direct methanation of CO is rather infrequent due to complete inactivation of methanogens in the presence of moderate concentrations of CO [76, 77]. Four methanogenic species have been demonstrated to use CO for growth: *Methanothermobacter thermautotrophicus* [106], *Methanosarcina barkeri* [107], *Methanosarcina acetivorans* [108, 109], and *Methanobacter marburgensis* [110]. However, all of them require rather long periods of adaptation to CO and growth is significantly slower than on their typical substrates. Consequently, CO conversion to methane in microbial communities is highly dependent on bacterial–archaeal interactions. Several studies have demonstrated the preferential use of certain pathways in anaerobic sludges used for syngas biomethanation. In this regard, the incubation temperature plays a determining role. Experiments with vancomycin, an inhibitor of acetogenic activity, have shown that, under mesophilic conditions, methanogenesis occurs primarily via acetate as intermediate [74, 93]. Mesophilic conditions are favourable to acetogenic bacteria, which provide acetate to acetoclastic methanogens; in contrast, higher temperatures generally shift the microbial community towards H_2-producing carboxydotrophs, which favour the hydrogenotrophic methanogenic route [76, 77, 92]. In both environments, when the H_2 pressure is kept sufficiently low, acetate can be converted to H_2/CO_2 by syntrophic acetate-oxidising bacteria, which can compete with acetoclastic methanogens and create a niche for hydrogenotrophic methanogens [78, 93]. The effect of temperature on the microbial composition of communities performing syngas biomethanation extends to the process performance. Several studies have reported the positive impact of thermophilic over mesophilic conditions on

conversion rates [59, 61, 76, 77, 92]. For example, Grimalt-Alemany et al. [92] observed an 18-fold higher methane productivity from enrichments incubated at 60°C compared to enrichments incubated at 37°C. Thermophilic conditions are therefore the preferred mode of operation for syngas biomethanation processes [84, 85].

Two other operational conditions, the pH and the pCO, also influence the performance of methanogenic communities using syngas. Since most methanogens grow optimally around neutrality, syngas biomethanation processes are generally operated at pH values between 7 and 7.6 [84, 85]. Carboxydotrophic bacteria can also proliferate in this pH range [27], thus providing intermediates for methanogenesis. The effect of CO levels has been extensively studied. Alves et al. [73] reported no methane production in thermophilic enrichments from anaerobic sludge incubated with solely CO as substrate (35 kPa). Methane was detected in enrichments incubated with $CO/H_2/CO_2$ ($pCO = 18$ kPa), but production ceased in subsequent transfers. Instead, both syngas- and CO-enriched cultures produced acetate. Elimination of methanogens in the enrichments could be due to the low growth rate of methanogens or their higher susceptibility to CO [77, 109, 110]. Similarly, Luo et al. [111] reported 50% lower methanogenic activity by an anaerobic sewage sludge exposed to a pCO of 51 kPa, compared to the control in the absence of CO. These observations are in line with those of Guiot et al. [77], who reported inhibition of methanogenesis in enriched granular sludge at pCO between 30 and 83 kPa. Nevertheless, some archaeal genus, such as *Methanobacterium*, have been shown to tolerate CO levels up to 96 kPa in microbial communities [83]. In addition, strategies are in place to enhance CO utilisation by methanogenic cultures. For instance, a system with gas recirculation enabled a CO conversion efficiency of 75% and a methane yield of 95% under a pCO of 60 kPa [77]. In some cases, an acclimation phase has enabled methane production by anaerobic sludges exposed to 100 kPa CO [93, 112].

Syngas biomethanation has been investigated in a variety of process configurations with the aim to improve gas-to-liquid transfer and cell concentrations. Besides the use of traditional stirred-tank reactors (CSTRs), tested designs include bubble columns, gas-lift reactors, trickle-bed reactors (TBRs) and multi-orifice baffled bioreactors (MOBBs) [84, 85]. The most promising results so far have been obtained with the use of TBRs. Recently, Asimakopoulos et al. [59] reported a CH_4 productivity from syngas of 8.49 $mmol \cdot L_{bed}^{-1} \cdot h^{-1}$ in a lab-scale TBR operated in continuous mode at 60°C. The inoculum used was an enriched mixture of two anaerobic sludges, intended to increase microbial diversity. Interestingly, the methanogenic population was more abundant in the biofilm of the TBR, while carboxydotrophic bacteria were mostly found in the liquid phase. In a follow-up study, the process was scaled up; a 7.5 L TBR, that used the same enrichment and syngas mixture and was operated in the same conditions as the lab-scale reactor, achieved a maximum CH_4 productivity of 17.6 $mmol \cdot L_{bed}^{-1}\ h^{-1}$, the highest reported so far for syngas biomethanation [60]. At this rate, H_2 and CO conversion efficiencies were 97% and 76%, respectively, and CH_4 selectivity was 99%. The higher performance of the scaled-up system was attributed to an improved gas-liquid mass transfer due to a

more efficient sparging component and a much higher height/diameter ratio, in addition to a more accurate pH control. To further test the process, the TBR was coupled to a gasifier that generated syngas from wood pellets; the generated gas contained 11–27% CO and was fed into the reactor at atmospheric pressure. The microbial consortium produced CH_4 at a maximum rate of 14.4 mmol·L_{bed}^{-1} h^{-1} without any inhibitory effects [60]. Few other studies have demonstrated continuous operation of bioreactors for syngas biomethanation by open cultures with syngas ($CO/H_2/CO_2$) as sole substrate [60, 84, 85]. Pereira [63] studied syngas conversion to methane by a mesophilic sludge in a 10.6 L MOBB operated in continuous mode. The system produced CH_4 at a maximum rate of 73 mmol L^{-1} day^{-1} with negligible amounts of by-products in the liquid and a $Y_{CH4/CO}$ of 0.6–0.8 (mol/mol), higher than reported in similar works [84, 85]. Yet, the conversion efficiency eventually dropped due to the prolonged high flow rates applied [63]. In a recent study, Chandolias et al. [62] tested a novel configuration consisting of a floating membrane in a membrane bioreactor and achieved a maximum CH_4 productivity of 34 mmol L^{-1} day^{-1}, in this case, using a thermophilic digester sludge.

Overall, CH_4 productivity in syngas biomethanation processes is very dependent on the process configuration and specific process conditions, which affect gas-to-liquid mass transfer and cell concentrations. Considerable progress over the last years and successful examples of scale-up cases such as that of Asimakopoulos et al. [60] offer good perspectives for syngas biomethanation in the future. A key aspect to bring this technology to commercial application will be to ensure its economic feasibility by, e.g., combining syngas biomethanation with existing gasification plants and improving reactor design to increase productivities [84, 85].

2.3 *Production of Ethanol*

Ethanol is undoubtedly the most common target product of syngas fermentation due to its commercial use as biofuel [16, 104, 113]. Despite high productivities have been achieved with pure cultures of acetogens, the robustness of open mixed culture operation has driven an interest for its production in these systems. Singla et al. [80] were the first to demonstrate ethanol production by microbial communities using syngas. In their study, a mesophilic enriched consortium obtained from faeces produced up to 2.2 g L^{-1} ethanol in semi-continuous mode (adding fresh syngas to serum bottles every 24 h). Liu et al. [69] tested continuous fermentation of syngas to ethanol by mixed culture in a CSTR including a cell recirculation unit. Authors reported the production of up to 8 g L^{-1} ethanol from syngas at 37°C and pH 7. Consumption of ethanol was followed by the accumulation of propanol and butanol, with peak concentrations of 6 g L^{-1} and 1 g L^{-1}, respectively. The microbial community was composed of the alkaliphilic acetogen *Alkalibaculum bacchi* (56%), the propionibacterium *Anaerotignum propionicum* (formerly, *Clostridium propionicum*; [114]) (34%) and other Clostridia species (10%). A follow-up study concluded that the mixed culture could convert 50% more carboxylic acids into their

respective alcohols compared to monocultures of *A. bacchi* [115], evidencing the positive effect of synergistic microbial interactions in syngas-fermenting communities.

A limitation of the fermentation process of Liu et al. [69] was the rather low CO and H_2 utilisation (20–60%), a common problem due to the low solubility of these gases and low gas mass transfer rates in CSTRs. Novel reactor configurations can help overcome this issue [116]. In a recent study, Wang et al. [67] reported a relatively high ethanol production from syngas by mixed culture in a hollow-fibre membrane biofilm reactor (HfMBR). HfMBRs, most popular in the field of gas and wastewater treatment, have recently attracted the attention of researchers in the field of syngas fermentation [64, 67, 116, 117]. In a HfMBR, a gaseous substrate flows through the lumen of a hollow-fibre membrane and is consumed by the biofilm formed on the outer surface of the membrane. The high surface area allows a high volumetric gas transfer rate which, in turn, translates into high production rates [118]. A non-acclimated sludge used by Wang et al. [67] produced up to 16.9 g L^{-1} ethanol from CO/H_2 (60:40) in a HfMBR operated in consecutive batch at pH 4.5 and 35°C. Ethanol was the only soluble product of CO/H_2 fermentation. Interestingly, a similar HfMBR-based process operating at pH 6.5 and 55°C converted CO/H_2 (40:60) to mostly acetate (98.6%) [64]. While the different temperature, gas composition and sludge characteristics could have contributed to the divergent product profile observed in these two studies, pH is most likely the determining factor. Several studies have reported that an acidic pH is key to promote ethanol production in syngas fermentation cultures [71, 72, 84, 85, 119]. Yet, no ethanol (but acetate) was produced by a sludge-derived culture in a HfMBR operated at pH 4.5 using H_2/CO_2 as substrates [120], highlighting that CO, which is a stronger reductant than H_2, is also essential to promote alcohol production by acetogens.

2.4 Production of Carboxylic Acids and Higher Alcohols

Acetate is the simplest carboxylic acid that can be produced from syngas. Titres in the range of 20–30 g L^{-1} have been obtained for sludge-derived consortia utilising syngas in continuous fermentation [64, 96]. Continuous operation of a thermophilic HfMBR reached a maximum acetate production rate of 16.4 g L^{-1} day^{-1} with high product selectivity [64]. However, acetate production by mixed cultures has not received much attention due to the significantly higher production rates that can be obtained by pure cultures and the rather low economic value of this product.

Instead, over the last decade, increased attention has been given to the production of medium-chain carboxylic acids (MCCAs) via anaerobic fermentation processes, a type of biorefinery referred to as the carboxylate platform [121–124]. The carboxylate platform relies mostly on sludges used in classical anaerobic digestion and aims at revalorising wastewater streams. Recently, several studies have extended this platform to the revalorisation of CO-rich gases. The products of syngas fermentation, acetate and ethanol, can be used by microorganisms that perform ethanol-based

chain-elongation, producing MCCAs such as butyrate and caproate as end-products. Different processes are devised to convert syngas into MCCAs by microbial communities, including the use of synthetic co-cultures, discussed in Sect. 3.3, and multiple-step processes, summarised elsewhere [58]. Here, the focus is on one-step conversions by open mixed cultures.

Ethanol chain-elongating communities are present in both natural and engineered environments and are dominated by relatives of *Clostridium kluyveri*, the best-studied ethanol chain-elongating microorganism and only isolate to date [122, 124, 125]. Mesophilic conditions are preferred to *C. kluyveri* and most acetogens. However, the two isolated strains of *C. kluyveri* grow optimally at a pH of 6.8 and 7.6 [126, 127], while acetogenic bacteria generally thrive in mildly acidic conditions [27, 128]. Therefore, pH is critical in determining the outcome of syngas-fermenting chain-elongating communities [70, 71, 129].

Ganigué et al. [70] used a carboxydotrophic enrichment from sludge in a syngas fermentation process without pH control. Acetogenesis was dominant at the initial pH of 7, while production of C4-C6 compounds prevailed at the mid/end of the fermentation, when the pH dropped to ~4.3. At the end of the process, the C4-C6 products represented 75–90% of the total, with butyrate (2.17 g L^{-1}) as main product. In a follow-up study, it was determined that pH values around 4.8 favoured a sustained production of higher alcohols [71]. In a semi-continuous process without pH control (initial pH 6), the mixed culture converted syngas to a maximum of 1.1 g L^{-1} butanol and 0.6 g L^{-1} hexanol. To favour the synthesis of C6 compounds, attributable to *C. kluyveri*, it was critical to prevent pH to decrease below 4.5–5.

In a recent study, He et al. [65] used a novel reactor configuration to promote gas transfer in a chain-elongating process. The system consisted of a reactor filled with a porous sponge pad and with a gas recirculation line. CO was used as sole carbon and energy source, and the partial pressure was gradually increased through the fermentation, from 15 to 61 kPa CO. Similar to the studies of Ganigué et al. [70, 71], operation was done at mesophilic conditions and the pH, initially set at 7, was not controlled. In contrast to those studies, though, the culture did not produce alcohols but a mixture of odd- and even-chain carboxylic acids including caprylate (C8), detected for the first time in a syngas fermentation process by a mixed culture. Production of C6-C8 carboxylates only began at the end of the fermentation, when CO pressure was 61 kPa. Maximum concentrations of caproate, heptanoate and caprylate were 0.22 g L^{-1}, 0.21 g L^{-1} and 0.15 g L^{-1}, respectively. The production of C5-C8 carboxylates halted in the last phase, likely due to product inhibition. The different product profile compared to the studies of Ganigué and co-workers could be explained by the different inoculum source and enrichment process, which resulted in quite different microbial compositions of the enriched cultures. The consortium used by Ganigué et al. [71] was mainly composed of *C. ljughdalii*, *C. carboxidivorans* and *C. kluyveri*, while He et al. [65] enriched a microbial community dominated by species of *Acinetobacter*, *Alcaligenes*, *Rhodobacteraceae* and a low abundance of *Clostridium spp*. On the other hand, product concentrations and production rates achieved by He et al. [65] were not higher than those reported in similar studies. This could be due to (1) the use of a non-acclimated inoculum, (2) the

use of CO (not syngas) as sole carbon and energy source and (3) CO provided ($\leq$60 kPa CO) being insufficient. Regardless, this work demonstrated that up to C8 carboxylic acids can be produced from CO in one-pot cultivation.

Similar to ethanol production, the use of HfMBR has proven very promising for the production of carboxylic acids from syngas. Shen et al. [64] demonstrated production of MCCAs from CO/H_2 for the first time in a HfMBR. Cultivation of the sludge-derived culture was studied at pH 6 under mesophilic (35°C) and thermophilic (55°C) conditions. In both scenarios, utilisation of CO and H_2 exceeded 95%. Mesophilic cultivation in sequential batch mode produced caproate (0.88 g L^{-1}) and up to 0.53 g L^{-1} caprylate, the highest caprylate concentration reported for a CO-fermenting system using mixed cultures. In contrast, thermophilic batch cultivation yielded a high acetate concentration (27.9 g L^{-1}) and product specificity (96.7%), with butyrate (<1 g L^{-1}) as only elongated carboxylate. Microbial community analysis revealed that the mesophilic enrichment was abundant in *Clostridium spp.* (41.6%), while the thermophilic microbial community comprised a large proportion of close relatives to *Thermoanaerobacterium* (92.8%). Overall, the work of Shen et al. [64] highlighted the great potential of HfMBR in syngas/CO fermentation processes and its functionality with open mixed cultures.

In a recent study, Chakraborty et al. [72] applied a cycle of high and low pH cycle to produce MCCAs in a stirred-tank reactor using a CO-acclimatised sludge. CO was used as sole substrate. The system produced acetate (6.2 g L^{-1}), butyrate (1.2 g L^{-1}) and caproate (0.42 g L^{-1}) at pH 6.2, which were subsequently converted to ethanol (11.1 g L^{-1}), butanol (1.8 g L^{-1}) and hexanol (1.5 g L^{-1}) at pH 4.9. The concentrations of butanol and hexanol obtained in this study are the highest reported so far for mixed cultures growing on syngas. However, CO utilisation remained below 59%, very far from the high conversion efficiencies obtained in HfMBR systems [64, 120].

3 CO Conversion by Synthetic Co-cultures

3.1 Synthetic Co-cultures: A Win-Win

A fundamental principle of bioprocess design is that a microorganism should be selected that fits the right process, not the other way around [130]. Synthetic co-cultures offer the possibility to select and combine microbial strains harbouring the required pathways for a specific process without renouncing some of the benefits of mixed cultures, such as robustness or division of labour [41, 42]. Contrary to open mixed cultures, microbial species that contribute negatively to the consortium (e.g., by generating undesired by-products, "stealing" intermediates or hampering growth of other partners) can be left out of the consortia. This cultivation approach has a big potential in the field of syngas fermentation. By using synthetic co-cultures, metabolic networks can be constructed that yield a broader range of end-products compared to monocultures of acetogens. In addition, such networks can be translated

into multi-compartment kinetic and genome-scale metabolic models, contributing to a further understanding of the microbial interactions and to optimisation of the system [131–133]. Computational modelling can also be used to predict novel co-culture interactions [134].

Yet what makes synthetic co-cultures stand out over other cultivation approaches is their unique feature: modularity [42]. Microbial strains can be incorporated or removed from an established consortium to fit a different process. In principle, there is only one imperative when designing co-cultures for syngas fermentation: at least one of the partners must be able to grow on CO. The choice of the other partner (s) will largely determine the product spectrum of the co-culture. To date, synthetic co-cultures have been established to convert CO/syngas into a range of products including methane, carboxylic acids, higher alcohols and other added-value chemicals. The following sections relate such developments, which are summarised in Table 2.

3.2 Production of Methane

Methane can be produced from syngas by mixed cultures, as discussed earlier (Sect. 2.2). A drawback of using these cultures is that competing pathways result in the production of side products such as acetate, propionate, ethanol or methanol, overall lowering methane specificities and production rates [77, 84, 85]. An alternative is to employ defined consortia that selectively combine efficient carboxydotrophic strains with CO-tolerant methanogens that can use the products of syngas fermentation at high rates. The conversion of CO to methane via H_2/CO_2 is preferred to the acetate route, as it results in higher conversion rates [77]. Several hydrogenogenic carboxydotrophs have been isolated [24]; among them, the thermophile *Carboxydothermus hydrogenoformans* and the photosynthetic mesophile *Rhodospirillum rubrum* are well-studied and have been employed in synthetic co-cultures for syngas conversion to methane.

Klasson et al. [135] established a methanogenic co-culture composed of *Rhodospirillum rubrum* and two methanogens, *Methanosarcina barkeri* and *Methanobacterium formicicum*. *R. rubrum* grows anaerobically on CO in the presence of light, producing H_2 and CO_2. The methanogens are both capable of H_2/CO_2 conversion to methane, with some differences. *M. formicicum* displays a high rate of H_2 uptake but is inhibited in the presence of CO. On the other hand, *M. barkeri* has higher tolerance to CO but converts H_2 at a lower rate. To compensate for each other's weaknesses, both methanogens were co-cultivated with *R. rubrum* to increase conversion of syngas to methane. The performance of the tri-culture was studied in two reactor configurations: a packed bubble column (PBC) and a TBR. Both reactors were operated under mesophilic conditions and included a light source to facilitate growth of *R. rubrum*. A 100% CO conversion was obtained in the TBE, while performance of the PBC was poorer, with a 79% CO conversion. Methane productivities reached 3.4 $mmol \cdot L_{liquid}^{-1}\ h^{-1}$ and 0.4 $mmol \cdot L_{liquid}^{-1}\ h^{-1}$ for the

Table 2 Overview of syngas fermentation processes using synthetic co-cultures

Microorganisms	Cultivation system	*T* (°C)	pH	Syngas composition (% v/v)	Product titre/productivity	Reference
Rhodospirillum rubrum, Methanosarcina barkeri and *Methanobacterium formicicum*	PBC	34	6.8–7.2	$CO/H_2/CO_2/Ar$ (55:20:10:15)	Methane 3.4 mmol L^{-1} h^{-1}	Klasson et al. [135]
Clostridium hydrogenoformans and *Methanothermobacter thermoautotrophicus*	CSTR	65	7.2	CO/H_2 (33:66)	Methane 6 mmol L^{-1} h^{-1}	Diender et al. [105]
Carboxydocella thermautotrophica, *Carboxydocella sporoproducens* and three methanogens	Serum Tubes	63	6.5	$CO/H_2/CO_2/CH_4$ (20:50:20:10)	Methane 0.72 mmol L^{-1} h^{-1}	Kohlmayer et al. [136]
Clostridium autoethanogenum and *Clostridium kluyveri*	Serum Bottles	37	6	CO/H_2	Butyrate 0.75 g L^{-1} day^{-1} Caproate 0.29 g L^{-1} day^{-1}	Diender et al. [137]
Clostridium autoethanogenum and *Clostridium kluyveri*	CSTR	37	6.2	CO/H_2 (47:53)	Butyrate 1.1 g L^{-1}, 0.55 g L^{-1} day^{-1} Caproate 0.82 g L^{-1}, 0.41 g L^{-1} day^{-1}	Diender et al. [138]
Clostridium ljungdahlii and *Clostridium kluyveri*	CSTR	37	6	$CO/H_2/CO_2$ (60:35:5)	Butyrate[a] 2.8 g L^{-1}, 2.7 g L^{-1} day^{-1} Caproate[a] 1.4 g L^{-1}, 1.3 g L^{-1} day^{-1} Hexanol[a] 4.7 g L^{-1}, 0.54 g L^{-1} day^{-1}	Richter et al. [129]
Clostridium ljungdahlii and *Clostridium kluyveri*	CSTR	37	6.3	$CO/H_2/CO_2$ (60:35:5)	Octanol[b] 0.78 g L^{-1}, 2 mg L^{-1} day^{-1}	Richter et al. [129]
Acetobacterium wieringae strain JM and *Anaerotignum neopropionicum*	Serum Bottles	30	7–7.2	$CO/CO_2/N_2$ (50:20:30)	Propionate 1.8 g L^{-1} Isovalerate 0.41 g L^{-1}	Moreira et al. [139]
Eubacterium limosum and *E. coli* GM	Serum Bottles	37	7	CO/N_2 (50:50)	3-HP 45.7 mg L^{-1} Itaconic acid 25.8 mg L^{-1}	Cha et al. [140]

PBC packed bubble column, *CSTR* continuous stirred-tank reactor, *GM* genetically modified, *3-HP* 3-hydroxypropionic acid

[a]Production rates are calculated as combined net rates in the reactor and in the gas stripping system. Butyrate and caproate concentrations refer to amounts dissolved in the reactor. Hexanol concentration refers to the condensate of the gas stripping system

[b]Octanol concentration and production rate refer to the condensate of the gas stripping system

TBR and the PBC, respectively. These are inferior to those reported for biological processes developed in later studies [60]. In addition, methane content in the outflow gas was rather low, 18–32%. Nevertheless, the work of Klasson et al. [135] demonstrated the functionality of synthetic co-cultures combining carboxydotrophic bacteria with methanogenic archaea for syngas biomethanation, establishing a precedent for later studies.

A more successful case is the thermophilic co-culture consisting of *Carboxydothermus hydrogenoformans* and the methanogen *Methanothermobacter thermautotrophicus* [105]. *C. hydrogenoformans* grows on CO producing H_2 and CO_2 as main products. *M. thermoautotrophicus* uses CO very poorly; however, it can tolerate its presence while growing on H_2/CO_2. Both strains grow optimally at around 65°C, with the advantage that such thermophilic conditions increase reaction kinetics and gas transfer rates [77]. Batch co-cultures of *C. hydrogenoformans* and *M. thermoautotrophicus* incubated at 65°C converted syngas to methane reasonably fast: 60 kPa CO were used in 24 h, while monocultures of the methanogen required 500 h to consume less CO. This suggests that removal of H_2/CO_2 by the methanogen favour thermodynamics of CO consumption by the carboxydotroph. Pressures up to 150 kPa CO could be used by the co-culture, which converted CO to CH_4 with $Y_{CH4/CO}$ close to theoretical values. In a CSTR, the co-culture of *C. hydrogenoformans* and *M. thermoautotrophicus* generated a headspace with methane peaks of 77% and a maximum production rate of 6 $mmol \cdot L_{liquid}^{-1}$ h^1. This is significantly higher than rates reported for similar processes with biomass retention [84, 85, 141], but still lower than the highest rate obtained in TBRs by Asimakopoulos et al. [60].

The genus *Carboxydocella* is another group of thermophilic bacteria comprising strains with a carboxydotrophic hydrogenogenic metabolism [142, 143]. Kohlmayer et al. [136] established a synthetic consortium of five microbial species: *Carboxydocella thermautotrophica*, *Carboxydocella sporoproducens* and three methanogens isolated from an anaerobic digester sludge (taxonomy of the archaea was not specified in the study). The co-culture was used to produce methane from syngas in thermophilic conditions. By including several strains with the same type of metabolism, functional redundancy and robustness were improved in the consortium. The two *Carboxydocella* strains can grow on CO, but *C. sporoproducens* exhibits slower growth compared to *C. thermautotrophica*. *C. sporoproducens*, though, can better withstand conditions of CO deprivation due to the formation of spores. On the other hand, methanogenesis was strengthened by the combined activity of three archaeal strains. In serum bottles, the mixed culture consumed 99% of the CO and 100% of the H_2 and produced an outflow gas with a 55% CH_4 content, which was 100% of the theoretical maximum for the used syngas. Interestingly, the co-culture proliferated well without added vitamins or yeast extract, suggesting that the five microbes might benefit each other from nutrient exchange [136]. Despite this was a rather small-scale study, is showed the positive effect of functional redundancy in syngas-fermenting microbial communities and demonstrated the viability of a five-partner consortia, which is rather uncommon [57].

3.3 Production of Carboxylic Acids and Alcohols

Synthetic co-cultures are promising systems for the production of MCCAs from syngas. Microbial MCCA production using monocultures of wild-type acetogens is challenging. A few acetogenic strains have been reported to produce C4 and C6 compounds from $CO/H_2/CO_2$, among them *C. carboxidivorans* [144] and *E. limosum* [145], yet in rather low concentrations. A popular alternative is the co-cultivation of an acetogen with *C. kluyveri*, whose unique metabolism is based on the elongation of short-chain carboxylic acids with ethanol or other electron donors [122, 124].

Diender et al. [24] established a synthetic co-culture of *C. kluyveri* and the well-known acetogen *C. autoethanogenum* that produced butyrate and caproate as end-products from solely CO as carbon and energy source. In this system, *C. autoethanogenum* converts CO into acetate and ethanol, which are taken up by *C. kluyveri* to produce butyrate and caproate. Butanol and hexanol were also detected, resulting from the reduction of the respective carboxylates by *C. autoethanogenum*. Experiments showed that, while growth of *C. kluyveri* was inhibited in the presence of >50 kPa CO in monocultures, it was sustained under a headspace of 130 kPa CO in co-cultivation with *C. autoethanogenum*. This co-culture illustrated how mutualistic interactions can be exploited to establish robust synthetic co-cultures. *C. autoethanogenum* produces the substrates for *C. kluyveri* (acetate and ethanol), at the same time that keeps dissolved CO levels low enough to allow growth of its partner. In addition, it was observed that ethanol production in monocultures of *C. autoethanogenum* would not have been sufficient to support growth of *C. kluyveri*. A follow-up study suggested that *C. kluyveri* enhanced solventogenic metabolism of *C. autoethanogenum* by removing ethanol from the environment [138]. Gene transcription of the central metabolism of *C. autoethanogenum* did not change in co-culture compared to monoculture conditions, indicating that the metabolic shift in the presence of *C. kluyveri* was thermodynamically driven. This is in line with related studies supporting that acetogenesis/solventogenesis in gas-fermenting microorganisms is controlled at the thermodynamic level [119, 146]. In continuous fermentation, the co-culture of *C. autoethanogenum* and *C. kluyveri* produced butyrate and caproate at rates of 0.55 g L^{-1} day^{-1} and 0.41 g L^{-1} day^{-1}, respectively. This work, established as proof-of-concept, was recently picked up by industry in a joint project of the corporations Evonik and Siemens, demonstrating the great potential of syngas-fermenting synthetic co-cultures [147].

Richter and colleagues upgraded the synthetic co-culture approach of Diender et al. [137] with a continuous bioprocess that included cell-recycling and in-line product extraction [129]. The co-culture was established with *C. kluyveri* and *C. ljungdahlii*, a close relative of *C. autoethanogenum* with excellent ethanol productivities from syngas [128]. Similar to the co-culture of Diender et al. [137], in this consortium *C. ljungdahlii* produced acetate and ethanol from syngas, which were used by *C. kluyveri* to produce longer-chain carboxylates via chain-elongation. In

addition, at a narrow pH range of 5.7–6.4, the elongated carboxylates were reduced by *C. ljungdahlii* to their respective alcohols, n-butanol, n-hexanol and, for the first time in a syngas-fermenting system, n-octanol was detected (up to 0.78 g L^{-1} in the condensate of the gas stripping system) [129]. Operation of their bioreactor at pH values higher than 6.4 gradually reduced and eventually crashed the population of *C. ljungdahlii*, which requires mildly acidic conditions to support growth. Without the acetogen, ethanol production halts and the co-culture crashes. On the other hand, mildly acidic conditions are detrimental to *C. kluyveri*, which grows in a pH range of 6–7.5 [126, 127]. In addition, acidic pH values result in the accumulation of undissociated acids (p*K*a $\sim$ 4.7), which are toxic to microorganisms [148]. This discrepancy between optimum pH for solventogenesis and acid production has been reported in similar studies [71, 137]. As Richter and co-workers stated, there is a need to isolate chain-elongating microorganisms with an optimum pH of growth of 5–5.5, a more favourable environment for acetogens to produce ethanol [129].

Alternatively, carboxydotrophic strains could be employed that can thrive at a pH range around neutrality. While the most prominent carboxydotrophic strains thrive in mildly acidic conditions [128], acetogens have been isolated with optimal pH values ranging from 5.4 to 9.8 [27]. An example is *Acetobacterium wieringae* strain JM, a novel carboxydotroph that grows optimally at pH 7 [112]. The authors of that study speculated that *A. wieringae* strain JM played a crucial role in syngas-enriched communities by providing substrate to ethanol-consuming propionibacteria, which grow optimally at neutral pH [149]. To test this hypothesis, Moreira et al. [139] cultivated *A. wieringae* strain JM with *Anaerotignum neopropionicum*, a propionibacterium unique for its ability to grow on ethanol [150]. The synthetic co-culture was capable of converting CO to propionate (1.78 g L^{-1}) via cross-feeding of ethanol at pH 7. In addition, isovalerate was detected in low amounts in the co-culture, while not in monocultures. Isovalerate could be produced by *A. neopropionicum* from amino acids; therefore, authors hypothesised that amino acid transfer took place between *A. wieringae* and *A. neopropionicum* in co-culture. Interestingly, proteomic analysis of the co-culture revealed sign of stress response in both strains, such as increased abundance of sporulation and antibiotic resistance proteins. It remains a question whether this would negatively affect the stability and functionality of the co-culture in the long-term or, on the contrary, the two populations would eventually come to a beneficial deal.

3.4 Production of Other Value-Added Chemicals

So far, this chapter has discussed various case studies of synthetic co-cultures and open mixed cultures employed in the conversion of syngas/CO to commodity chemicals such as methane, MCCAs and simple alcohols. The production of biochemicals of higher complexity by syngas-consuming cultures is still challenging, for example due to metabolic limitations of the microorganisms or to the requirement of different environmental conditions. A strategy that has been used to overcome this

issue is the introduction of engineered strains of genetically accessible microorganisms.

The majority of studies on the use of synthetic co-cultures applied to syngas fermentation have relied on the abilities of wild-type microbial strains [52, 105, 110, 129, 136]. Engineering of Clostridia strains to produce heterologous compounds has advanced rapidly in the last decade, but limitations remain and further understanding of molecular mechanisms is required [37]. Model microorganisms such as *Escherichia coli*, in contrast, have been widely engineered for the production of a vast range of biochemicals. If a suitable strategy is in place, such strains could be co-cultivated with acetogens to facilitate the production of valuable biochemicals from syngas. Cha and co-workers followed this approach with two co-cultures of the acetogen *E. limosum* grown with two genetically engineered *E. coli* strains [140]. In both co-cultures, *E. limosum* converted CO into acetate, which was used as carbon source by *E. coli*. The two engineered strains of *E. coli* used acetate to produce 3-hydroxypropionic acid (3-HP) and itaconic acid (ITA), respectively. At the end of batch cultivations (72 h), the co-cultures produced a maximum of 45.7 mg L^{-1} 3-HP and 25.8 mg L^{-1} ITA. Consumption of CO increased 10% in co-cultivation compared to monocultures of *E. limosum*, evidencing that the mutualistic interaction enhanced carbon flux. This study demonstrated for the first time the production of value-added chemicals (3-HP and ITA) from syngas using co-cultures. However, several issues need to be addressed, as noted by the authors. First, the concentration of CO dissolved had to be minimised to allow growth of *E. coli*, thus a condition of mass-transfer limitation was sought. This was initially achieved by inoculating *E. limosum* and *E. coli* at high ratios, up to 150:1 (based on OD_{600}). Over the course of cultivations, though, CO consumption rates and cell concentrations decreased, pointing to the need to improve process stability. Another major problem is that acetate assimilation by *E. coli* in anaerobic conditions requires the addition of an electron acceptor. Trimethylamine N-oxide (TMAO) was chosen since it yielded the highest acetate assimilation rate and it did not significantly affect CO conversion rates. The fact that TMAO should be supplied proportionally to the desired amount of product would significantly reduce co-culture efficiency and increase process costs, making it unfeasible to implement this strategy at industrial scale. Nevertheless, the work of Cha et al. [140] is a first step towards modular pathway engineering of synthetic co-cultures to facilitate the production of high-value chemicals from syngas.

4 CO Conversion via Sequential Processes

Sequential processes can also be used to produce value-added biochemicals from syngas. While these are not one-pot strategies, they may comply with the feature of modularity, characteristic of synthetic cultures. The greatest advantage of this approach compared to one-pot cultures is that it circumvents cultivation divergences between partners in a consortium, by growing each partner in a separate bioreactor

(e.g., combinations aerobic/anaerobic, low/high pH or temperature, etc.). A few proof-of-concept studies have shown the potential of this strategy applied to the conversion of CO-rich gases. Hu et al. [151] designed a two-stage process to produce microbial oil from syngas. In the first reactor (60°C, pH 6), the acetogen *Moorella thermoacetica* converted $CO/H_2/CO_2$ to acetate. Acetate was then fed into the second reactor (28°C, pH 7.3) for its aerobic conversion to C16-C18 triacylglycerides by an engineered strain of the yeast *Yarrowia lipolytica*. The integrated process produced 18 g L^{-1} lipids at a rate of 0.19 g L^{-1} h^{-1}. Acetate as intermediate was also used in the two-step process established by Oswald et al. [91] to produce malic acid. In this case, *C. ljungdahlii* was first grown in a batch reactor converting syngas to acetate. Subsequently, the reactor was adapted for aerobic cultivation of *Aspergillus oryzae*, which was inoculated on top of the existing culture. Malic acid was produced to a maximum concentration of 2.02 g L^{-1}. However, the second-stage process was not reproducible in triplicate reactors. Recently, the production of biopolymers has also been demonstrated using sequential processes [152, 153]. In one study, effluent from syngas fermentation by *C. autoethanogenum* (containing acetate, ethanol and 2,3-BDO) was fed in pulses into a second reactor for the production of polyhydroxyalkanoates (PHAs) [153]. The second reactor contained an enriched mixed culture used in a previous process adapted to PHA production. Only acetate was consumed by the mixed culture, which accumulated a maximum of 24% PHA. Hwang et al. [152] designed a two-stage process for polyhydroxybutyrate (PHB) production differing from the rest in that formate, instead of acetate, was used as intermediate. In the first stage, the acetogen *Acetobacterium woodii* was used for conversion of syngas under optimised conditions for ~100% formate selectivity. The formate solution was concentrated and supplied in fed-batch mode into the second reactor, where formate was converted to PHB by genetically modified *Methylobacterium extorquens* AM1. All these studies require further improvements, mostly related to medium optimisation in the second reactor. Inadequate composition of ions and certain (toxic) components in the syngas fermentation effluent can have a detrimental effect on the non-acetogenic partner. Nonetheless, these works show that integrated bioprocesses are a feasible platform to convert gaseous substrates to biochemicals of added-value.

5 Challenges and Opportunities of Syngas-Fermenting Microbial Communities

By discussing syngas fermentation by mixed cultures, it becomes clear that there are many challenges, but also many opportunities, for future developments in the field. Open mixed cultures are very robust and resilient, offering good prospects for the production of methane and short-chain fatty acids, such as acetate and butyrate. The main challenge with open mixed cultures is product selectivity. A better understanding of microbial compositions and interactions, and the effect of varying process

parameters, is necessary. Knowledge on complex microbial communities converting syngas may also source inspiration for the creation of synthetic co-cultures as recently exemplified by Moreira et al. [139], where a co-culture producing propionate was constructed based on the microbial composition of an enriched culture. Compared to the enriched culture, the co-culture produced higher amounts of propionate, and side products (like methane) were eliminated. Open mixed cultures and laboratory enrichments may also lead to the isolation of new microorganisms, carboxydotrophs or others, with better characteristics for the construction of synthetic co-cultures. For example, Richter et al. [129] observed suboptimal performance of a co-culture of *C. ljungdahlii* and *C. kluyveri* due to a mismatch in the optimal pH of the two species. Isolation of (ethanol-driven) chain-elongators with lower optimal pH for growth would be useful for pairing with solventogenic acetogens during syngas fermentation. Such microorganisms are currently not available in culture collections. Currently, there are also only a limited amount of thermophilic carboxydotrophs isolated, and most of them exhibit a hydrogenogenic metabolism. Studying high-temperature adapted microbiomes (e.g., thermophilic anaerobic sludges, hydrothermal vents, etc.) could lead to discovering novel microbes and metabolisms. Thermophilic organisms could be used to produce volatile compounds, allowing their separation in the gas-phase and reducing streaming costs. Other environments, such as high salinity sediments, are also not well studied in regard to their potential to convert CO/syngas [154].

The first steps for co-cultivation of microbes for syngas fermentation are taken. Now, work can be done in two fronts: improvement of current co-cultures for the production of, e.g., MCFA and alcohols (higher titres, higher yields, higher carbon fixation, etc.) or the development of new co-cultures for the diversification of products. The improvement of co-culture systems can be aided by genome-based models (GEMs). These models describe the set of possible reactions by the microbes in the co-culture (based on their genomic content), including extracellular exchange of metabolites [131, 134, 155–157]. The GEM constructed by Benito-Vaquerizo et al. [131] describes growth of the syngas-converting co-culture comprising *C. autoethanogenum* and *C. kluyveri*, and predicts that succinate addition would improve the production of MCFAs. Experimental testing needs to be conducted to ascertain this, but this is an example of how GEMs can aid in the generation of hypothesis and eventually result in accelerated optimisation of co-cultures. Computational models can also be used to predict novel microbial interactions. In a recent study, Li and Henson [134] performed in silico simulations on 170 combinations of acetogen and butyrate-producing bacteria pairings. This led to the discovery of highly performing co-culture designs for syngas fermentation that could guide future experimental studies. Yet, reconstruction of GEMs, and especially their manual curation and experimental validation is still a time-consuming procedure, and applications of GEMs to co-cultivation are so far scarce [158].

Co-cultures are also suitable for the introduction of genetically engineered strains, e.g. to supress/overexpress the expression of certain genes [159], engineer symbiosis [160], create ‘artificial’ division of labour [161] or control populations of different strains [162]. Regarding syngas fermentations, up to date only wild-type acetogens

have been used to establish synthetic co-cultures but this could change soon with the recent developments on genetic engineering of autotrophic Clostridia strains able to convert CO [34, 35]. Carboxydotrophy can also be engineered in solventogenic Clostridia, as shown by heterologous expression of a carbon monoxide dehydrogenase in *Clostridium acetobutylicum* [163]. The urge to produce high-value chemicals from syngas is also putting the focus on engineering pathways for the assimilation of one-carbon compounds (e.g., glycine pathway) in *E. coli* strains that can natively produce value-added chemicals [164, 165].

6 Conclusion

Microbial communities have a tremendous potential in syngas fermentation processes, broadening the product spectrum beyond acetate and ethanol. Open mixed cultures are sustained by decades of research and industrial experience on the field of anaerobic digestion (of wastes/wastewaters), which can be transferred to the conversion of CO-rich gases to methane, MCCAs and alcohols. Synthetic co-cultures can enhance product selectivity, offer modularity and allow the use of kinetic and genome-scale metabolic models for further optimisation. Recently, genetically engineered strains of model organisms have been co-cultured with acetogens, enabling the production of added-value chemicals from C1 substrates. Industrial implementation of syngas-fermenting microbial consortia for the production of valuable biochemicals is not yet a reality. However, growing interest on the utilisation of C1-gases nurtured by major efforts undertaken over the last few years might certainly drive this platform forward faster than anticipated.

Acknowledgements The authors would like to acknowledge the Netherlands Science Foundation (NWO) (Project NWO-GK-07), the NWO Applied and Engineering Sciences (AGS) (Perspectief Programma P16-10), and the Netherlands Ministry of Education, Culture and Science (Project 024.002.002) for financial support.

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