The microbiology of Power-to-X applications

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Abstract

Power-to-X (P2X) technologies will play a more important role in the conversion of electric power to storable energy carriers, commodity chemicals and even food and feed. Among the different P2X technologies, microbial components form cornerstones of individual process steps. This review comprehensively presents the state-of-the-art of different P2X technologies from a microbiological standpoint. We are focusing on microbial conversions of hydrogen from water electrolysis to methane, other chemicals and proteins. We present the microbial toolbox needed to gain access to these products of interest, assess its current status and research needs, and discuss potential future developments that are needed to turn todays P2X concepts into tomorrow's technologies.

Keywords: Power-to-X, green hydrogen, microbial protein, microbial electrochemical technologies, P2G, P2X

INTRODUCTION

Global climate change, mainly caused by anthropogenic greenhouse gas (GHG) emissions, is one of the biggest societal challenges. Abatement thereof might be achieved by combining various measures including renewable energy, nuclear power, carbon capture and storage (CCS) as well as utilization (CCU), and increasing efficiency in energy use to reduce humankind's carbon footprint (Rogelj *et al.* 2016; Fawzy *et al.* 2020). These measures aim at limiting global warming to below 2 degrees Celsius compared to pre-industrial levels, as agreed in the Paris Agreement (UNFCCC 2015). An important mitigation strategy is to replace fossil sources used for energy, chemicals and fuels with renewable sources. In the last decades, the share of renewables has increased substantially, especially in the sector of electric power production. According to the analysis and forecast of the International Energy Agency (IEA 2020), photovoltaics (PV) and onshore wind turbines in many countries are currently the most economic sources of electric power. In the last decade, the cost of onshore wind and solar energy dropped by 70% and 90%, respectively (Harnisch and Morejón 2021). The global weighted average levelized cost of energy (LCOE) in 2019 was USD 0.068 kWh^{-1} and USD 0.053 kWh^{-1} for utility-scale solar PV and onshore wind power, respectively (IRENA 2020). Renewables are predicted to account for 95% of the net increase in global electric power capacity by 2025 (IEA 2020).

The biggest challenge is the integration of the most popular renewables, *i.e.* wind and solar power, into the electricity grid due to their intermittent nature. They cannot provide electric power on demand, and are highly dependent on diurnal rhythms and weather conditions. Thus, a certain overcapacity is required for satisfying demand (Wagner 2016). During windy and sunny periods, this overcapacity results in local or even grid-wide surplus electricity that cannot be utilized as it would cause grid instabilities. Different technologies can be employed to store or

divert such surplus electric power. Apart from well researched batteries or capacity limited pump storage plants, these include the conversion of electric energy into storable energy carriers, chemicals and nutrients. These conversion technologies are discussed under the umbrella of "Power-to-X" (P2X) with "X" denominating the different kinds of use (Fig. 1). A central approach of P2X is the electrolysis of water to hydrogen and oxygen, which is also covered by the term Power-to-Gas (P2G). Molecular hydrogen (H_2) is a keystone molecule of future energy strategies in many countries (Jensterle *et al.* 2019; Federal Ministry for Economic Affairs and Energy 2020). It can be used as an energy carrier ("vector") or as resource for various (bio)technological processes. Due to the recent cost decrease in the production of renewable electricity and further conversion to hydrogen gas through water electrolysis, hydrogen is expected to reach a production cost of about € 1.0 kg^{-1} H_2 by 2030–2040 (Van Wijk, Van der Roest and Boere 2017).

Storage and transportation of H_2 are difficult due to the relatively low energy density (Luo *et al.* 2012) and small molecular size of H_2, which can cause diffusion through the walls of pipes and tanks, leading to losses and causing also embrittlement of steel pipelines (Hafsi, Mishra and Elaoud 2018). In addition, there are technical regulations limiting the H_2 concentration in the infrastructure that was built for natural gas. An alternative solution is to convert H_2 into bigger molecules with higher energy density, such as ammonia (NH_3) or methane (CH_4). Alternatively, surplus electric power can be used for abiotic hydrogenations and hydrodeoxygenations of complex molecules (Harnisch and Morejón 2021).

Another approach to avoid losses and transportation costs is to convert H_2 to other value-added products using microbial synthesis, which is an important branch of electrobiorefineries (Harnisch and Urban 2018). These conversion strategies are called Power-to-Chemicals or

Power-to-Proteins, the latter is the case when the hydrogen-utilizing microbial biomass is used as protein source for food or feed (Fig. 1). Hydrogen is an excellent electron donor for chemolithoautotrophic microbial processes and, thus, can be employed for microbial capturing of CO_2. Besides the typical anaerobic chemolithoautotrophs, *i.e.*, homoacetogenic bacteria and hydrogenotrophic methanogenic archaea, CO_2 fixation can be accomplished by aerobic, facultative autotrophic hydrogen-oxidizing bacteria (so-called Knallgas bacteria) and by phototrophic bacteria.

Autotrophic microbial cell factories have a higher potential to be sustainable than systems based on microbial utilization of plant biomass. This is due to their higher energy conversion efficiency and the more direct conversion routes from substrate, *i.e.*, CO_2, to product. However, when excluding open reactor microbiomes, *e.g.*, hydrogenotrophic methanogens in anaerobic digestion (AD), the specific large-scale biotechnological application of hydrogen-utilizing autotrophs has been limited, so far. This is in contrast to the assumption that these microorganisms will play a crucial role in a future electrified bioeconomy (Claassens *et al.* 2016). Another approach for CO_2 fixation employing surplus electricity is the electrochemical conversion of CO_2 to, *e.g.*, formate, acetate (De *et al.* 2020), and CO for further synthesis (Haas *et al.* 2018). Especially the organic products can be metabolized by microorganisms for biomass production and biosynthesis of chemical compounds (Chen *et al.* 2012; Li *et al.* 2012; Hegner *et al.* 2019, 2020; Lo Faro *et al.* 2019; Möller *et al.* 2019; Herranz *et al.* 2020; Yan *et al.* 2020). The major source of CO today is still biomass gasification to obtain synthesis gas but recent developments in nanostructured catalysts offer a unique opportunity to boost the selectivity and activity of CO_2 conversion to CO (Overa *et al.* 2022). The utilization CO containing syngas opens new opportunities for medium-chain carboxylate and alcohol production (Baleeiro *et al.* 2019, Baleeiro *et al.* 2022). Currently

Siemens and Evonik have started a large project named Rheticus to produce chemicals from CO obtained by the electrochemical reduction of CO_2 (https://www.kopernikus-projekte.de/en/projects/rheticus).

Although P2X are often regarded as carbon capture and storage (CCS) technologies (Bui *et al.* 2018), such storage is only temporary until utilization/oxidation of the products (*i.e.*, fuel or feed/food). Therefore, the term carbon capture and utilization (CCU) is more appropriate for P2X technologies. Nevertheless, the integration of P2X technologies into the circular economy has an enormous potential for the reduction of GHG emissions.

Box 1: Taxonomy notes

The taxonomy of microorganisms has changed over time as concepts advanced, but using the correct taxonomic names is of utmost importance for meaningful comparisons. Taxonomy that is naming and classifying microorganisms is a dynamic discipline with changing rules. Denomination of many well-studied microorganisms underwent several changes from the first description to current status. A well-known example with high relevance to P2X is the hydrogen-oxidizing bacterium *Cupriavidus necator* (Makkar and Casida 1987). This species appeared under various names in publications, such as *Alcaligenes eutrophus*, *Wautersia eutropha, Hydrogenomonas eutropha*, and *Ralstonia eutropha* (Vandamme and Coenye 2004), but the currently valid name is *C. necator*. Another example is the genus *Methanothrix* that was renamed to *Methanosaeta* as a result of a dispute over the description of the first pure culture, and this name was used for decades. However, as a result of changing rules of the International Code of Nomenclature of Bacteria, the genus was renamed again to *Methanothrix* according to a decision of the Judicial Commission of the International Committee on Systematics of Prokaryotes (Tindall 2014). Nevertheless, in most but not all of the biotechnology-related literature, the heterotypic synonym *Methanosaeta* has been and still is used. To avoid confusion and for assuring coherence, we use the most recent names and not the one appearing in the cited literature, but comment thereon when needed for clarification.

In P2X, various biocatalysts, such as pure single cultures, co-cultures of few microorganisms, or even complex mixed cultures can be utilized. Open mixed cultures have clear advantages when complex substrates of changing quality are used (Kleerebezem and van Loosdrecht 2007; Schlembach *et al.* 2021), but in case of gaseous substrates, the application of pure single cultures and defined co-cultures can be more suitable (Diender, Parera Olm and Sousa 2021). The pros and cons of pure culture vs. mixed culture approaches are discussed in the sections describing particular P2X technologies. We also use the term microbiota when referring to the assembly of microorganisms in complex communities, while the term microbiome is used in a broader context. A microbiome includes the "theatre of activity" including the genetic information, product spectra and interaction of the microbiota with their environment, which actively form a dynamic and interactive micro-ecosystem integrated in macro-ecosystems (i.e., eukaryotic hosts or whole reactors in case of biotechnological systems) (Berg *et al.* 2020). Genetic engineering to improve the metabolic capabilities of these microorganisms or to utilize their genetic potential in other microorganisms serving as host for productive bioprocesses is promising, but requires challenging genetic modification of hydrogen-utilizing autotrophs, as discussed elsewhere (Claassens *et al.* 2016).

Recent review articles covered specific technological and economic aspects of P2X processes (Götz *et al.* 2016; Takors *et al.* 2018, Thema *et al.* 2019; Ahmed *et al.* 2021). Here, we provide an overview of the metabolic potential of bioreactor microbiomes and pure strains as promising biocatalysts for P2X by deciphering the underlying principles and potentials from a microbial perspective. We illustrate how various biochemical conversion processes of P2X allow combinations of different sectors within the bio-based circular economy. We also give an overview of the current advances in molecular biological techniques for the analyses of the

microbial communities in P2X systems for addressing the limitations of the process or help steering the process to achieve higher yields, more stable operation or faster start up.

POWER-TO-GAS

Using P2G, electric energy is converted into chemical energy stored in gaseous energy carriers, such as H_2 or CH_4 (Schiebahn *et al.* 2015). P2G technologies for the production of highly pure H_2-gas via water electrolysis are operated at commercial scale and were recently reviewed (Shiva *et al.* 2019; Vasconcelos and Lavoie 2019). A technical solution for obtaining pure hydrogen is additional membrane purification benefitting from the size difference of the two molecules. Hydrogen as energy carrier suffers from low volumetric energy density and technical limits of injection to the natural gas grid. Therefore, adaptation of the existing gas grid infrastructure or the construction of new infrastructure is necessary to enable the storage, transport and utilization of H_2 (Angelidaki *et al.* 2018; Topolski *et al.* 2022). To circumvent these challenges and considerable capital expenditures, H_2 can be converted to CH_4 by thermochemical methanation (also called catalytic methanation) or biological methanation (also called biomethanation) (Eq. 1). Both technologies differ, *e.g.*, in terms of methane production rates, necessary gas purities, and process conditions (pressure and temperature). Methane has a higher volumetric energy density compared to H_2 and is much easier to store and to transport. For both methanation reactions, CO_2 is required as carbon source (Eq. 1) (Buan 2018). Potential CO_2 sources are industrial sources, *e.g.*, natural gas power plants, coal power plants, cement production, petroleum refineries, iron and steel manufacturing, biorefineries or AD plants (Chu *et al.* 2019).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O;\ \Delta G^{\circ\prime} = -135.6\ \text{kJ mol}^{-1}\ CH_4 \qquad \text{Eq. 1}$$

Box 2: Terminology for methane production

The term methane evolution rate (MER) describes the production rate of CH_4 from H_2 and CO_2 (Rittmann, Seifert and Herwig 2012, 2015; Costa *et al.* 2013; Rittmann 2015; Abdel Azim *et al.* 2017; Lecker *et al.* 2017; Savvas *et al.* 2017; Mauerhofer *et al.* 2018; Rittmann *et al.* 2018; Nock *et al.* 2019; Rusmanis *et al.* 2019). As the term evolution in the context of biology refers to the change in the characteristics of species over generations as a result of mutation and natural selection, the terms methane production rate (MPR) or methane formation rate (MFR) are more appropriate. Further, hydrogen production rate (HPR) from water electrolysis is also in line with electrochemical nomenclature referred to as hydrogen evolution rate (Deutzmann and Spormann 2017; Aryal *et al.* 2018; Palacios *et al.* 2019). In this review, we use the terms MPR (Navarro *et al.* 2016; Kracke *et al.* 2020) and HPR (Costa *et al.* 2013; Kracke *et al.* 2020) to assure clarity.

Use of hydrogen for biogas upgrading

From a technical perspective, there are three variants of biogas upgrading, namely, *ex situ* biogas upgrading (Rittmann 2015), *in situ* biogas upgrading (Luo *et al.* 2012), and hybrid biogas upgrading (Kougias *et al.* 2017). The term *in situ* biogas upgrading was first coined in 2012 (Luo *et al.* 2012), while the term *ex situ* biogas upgrading was first used in 2015 (Rittmann 2015). *In situ* upgrading refers to the process of injecting hydrogen directly to AD reactors. Yet, its solubility is very low (1.6 mg L^{-1} in water at 25°C) and only restricted amounts of hydrogen can be effectively injected without compromising the proper functioning of the AD process (Angelidaki *et al.* 2018). This means that biogas is upgraded only at low efficiency or low rate. There are several conceivable explanations for this phenomenon. First, the bioavailability of H_2 is insufficient due to the poor solubility of H_2 in the liquid phase at ambient temperature and pressure (Jud *et al.* 1997). Second, the co-substrate CO_2 can become limiting (Szuhaj *et al.* 2016). Third, inhibition of the syntrophic oxidation of volatile fatty acids (VFA) can occur due to high H_2 partial pressure (Luo and Angelidaki 2013a), which may lead to further CO_2 limitation

(Wahid *et al.* 2019). Fourth, methanogenesis can be inhibited due to VFA accumulation. Fifth, the change in the carbonate buffer equilibrium as well as capacity, due to the CO_2 consumption, may lead to a pH increase (Luo *et al.* 2012; Szuhaj *et al.* 2016) and consequently process failure. This problem can be mitigated by either using a co-substrate (e.g., manure and whey) (Luo and Angelidaki 2013b) or active gassing CO_2 at a higher operating expense and energy need (Szuhaj *et al.* 2016). Finally, competition of methanogenic archaea with homoacetogenic bacteria can occur at high H_2 partial pressure (Agneessens *et al.* 2018). Nevertheless, successful implementation of *in situ* upgrading has been demonstrated by applying innovative membrane bioreactors and proper adaptation (Deschamps *et al.* 2021). The combination of *in situ* biogas upgrading and bioaugmentation with the mesophilic methanogen *Methanoculleus bourgensis* was successful, but failed at higher process temperature applied to the thermophilic strain *Methanothermobacter thermoautotrophicus* (Palù *et al.* 2022). Recent progresses in biogas upgrading using *in situ* technologies was reviewed recently, and H_2 addition by hollow fiber membranes and high-pressure anaerobic digestion in combination with bioelectrochemical system found to be the most promising technologies (Zhao *et al.* 2021).

The *ex situ* process means that external or recovered CO_2 from physico-chemical biogas upgrading units (Szuhaj *et al.* 2016), or even raw biogas is fed with hydrogen in a separate reactor to produce methane (Rittmann 2015). This process is catalyzed either by pure cultures (Rittmann, Seifert and Herwig 2015) or by a microbial community (Lee *et al.* 2012; Kim *et al.* 2013; Díaz *et al.* 2015, 2020; Mohd Yasin *et al.* 2015; Rachbauer *et al.* 2016, 2017; Szuhaj *et al.* 2016; Bassani *et al.* 2017; Dupnock and Deshusses 2017; Kim *et al.* 2017; Kobayashi *et al.* 2017; Kougias *et al.* 2017; Strübing *et al.* 2017, 2018, 2019; Angelidaki *et al.* 2018; Omar *et al.* 2018; Jensen *et al.* 2019; Maegaard *et al.* 2019; Figeac *et al.* 2020; Logroño *et al.* 2020).

The hybrid process combines *in situ* and *ex situ* biomethanation approaches (Kougias *et al.* 2017; Angelidaki *et al.* 2018; Corbellini *et al.* 2018) by partially upgrading biogas via injection of H_2 to the main reactor as first step, followed by a refining step to reach methane contents of >90-95% via *ex situ* upgrading in a separate reactor. Depending on the reactor type and mode of operation, biocatalysts exist in the form of planktonic cells or biofilms.

Planktonic cells are common in continuous stirred tank reactor (CSTR) systems, whereas biofilms are characteristic of fixed-bed reactors (FBR) (Savvas *et al.* 2017; Aryal *et al.* 2018; Rusmanis *et al.* 2019; Formann *et al.* 2020). There has been a growing interest in further developing the biological biogas upgrading, and previous studies investigated different reactor setups (Kougias *et al.* 2017; Formann *et al.* 2020), the flexibility of the bioprocess (Strübing *et al.* 2018, 2019), the feeding mode (Szuhaj *et al.* 2016), methods to improve gas delivery (Szuhaj *et al.* 2016; Bassani *et al.* 2017; Jensen *et al.* 2018, 2021a; Ghofrani-Isfahani *et al.* 2021), various packing materials in trickle-bed reactors (Daglioglu *et al.* 2021: 2; Dağlıoğlu *et al.* 2021: 2; Jensen *et al.* 2021b; Ghofrani-Isfahani *et al.* 2022), and effects of temperature (Figeac *et al.* 2020).

From the practical point of view, combining an external source of H_2 with the AD technology is advantageous. The gas feed is already oxygen-free and moisturized, and no additional CO_2 separation is necessary. Thus, increased efficiency of carbon utilization is reached as well as transformation of H_2 into an energy carrier, *i.e.*, CH_4 that is more compatible with existing supply and storage infrastructure.

Microbial communities in biological biogas upgrading

In AD, a cascade of microbial processes, characterized by specific functional traits of the microbiota, takes place during the degradation of organic substrates to biogas. AD is characterized by four stages, namely, hydrolysis, acidogenesis, acetogenesis (syntrophic VFA oxidation) and methanogenesis. The proper functioning of the AD process relies on the food web in the reactor, rather than on the activity of a single microorganism. Microorganisms in AD processes can be assigned to different functional groups according to their metabolic traits (**Table 1**).

The proper functioning of an AD process requires adequate functioning of the tightly linked acetogenesis and methanogenesis metabolic traits, which results in an obligately syntrophic relationship. Syntrophy involves a microbial trading process where energy carriers are the currency to ensure the energy gain for each of the syntrophic partners to thrive. Interspecies electron transfer refers to mechanisms of syntrophy between different microbes where hydrogen (interspecies hydrogen transfer), formate (interspecies formate transfer) or electrons (direct interspecies electron transfer (DIET)) are exchanged (Stams and Plugge 2009; Liu *et al.* 2012; Rotaru *et al.* 2014). The most well-known syntrophy in AD is the interaction between hydrogenotrophic methanogens and syntrophic acetate-, propionate- or butyrate-oxidizing bacteria. Hydrogenotrophic methanogens are equipped with hydrogenases (Thauer *et al.* 2010) and formate dehydrogenases and, due to their high substrate affinity and activity, keep the hydrogen or formate concentration extremely low (10 Pa and 10 μM, respectively). As a result, thermodynamically feasible conditions for the benefit of syntrophs that degrade VFA are created. Neither the methanogens nor the bacteria alone can thrive from the degradation of VFA (Schink 1997; Schink and Stams 2006; Stams *et al.* 2012; Wickham 2016). There are several known

genera of syntrophic VFA degraders (Schink and Stams 2006; Stams *et al.* 2012), but it becomes more and more evident that our current knowledge is only the tip of the iceberg, as thanks to omics methods new syntrophic species are continuously being discovered (Hao *et al.* 2020; Singh *et al.* 2021; Becker *et al.* 2023). Known genera of syntrophic acetate, propionate and butyrate oxidizers are summarized in **Table 1**. H_2 injection into AD reactors during *in situ* biogas upgrading can have severe consequences to the microbiota, as it disturbs the syntrophic VFA oxidation (H_2-production) and H_2 consumption by methanogens (Fukuzaki *et al.* 1990). High H_2 partial pressure can increase the abundance of hydrogenotrophic methanogens, but opens also niches for homoacetogenic bacteria that are known to compete for electrons and carbon to produce acetate (Angelidaki *et al.* 2018). Bioaugmentation of the mesophilic hydrogenotrophic strain *Methanoculleus bourgensis* in combination with external hydrogen addition resulted in increased biomethanation in a biogas process with cheese whey and manure as main substrates (Palù *et al.* 2022). In contrast, bioaugmentation failed in the same setup but at higher temperature with a thermophilic *Methanothermobacter thermautotrophicus* strain, and *Methanosarcina* became the dominant methanogen as it mainly utilized acetate produced by homoacetogenic bacteria from the supplemented hydrogen (Palù *et al.* 2022). Slow adaptation of the initial microbiome of a pilot-scale anaerobic digester to *in situ* biomethanation showed a shift from homoacetogenesis and acetoclastic methanogenesis to hydrogenotrophic methanogenesis (Deschamps *et al.* 2021). Using granular sludge from an upflow anaerobic sludge blanket (UASB) reactor as inoculum to perform *ex situ* biomethanation showed that *Methanobacteriaceae* had a selective advantage over other hydrogenotrophic methanogens (Logroño *et al.* 2021). Other studies have also found members affiliated to this family to be dominant in the microbial community performing biomethanation (Luo and Angelidaki 2012;

Kougias *et al.* 2017; Rachbauer *et al.* 2017; Savvas *et al.* 2017; Braga Nan *et al.* 2020, 2022). The thermophilic genus *Methanothermobacter* is the predominant methanogen in high temperature AD processes (Szuhaj *et al.* 2021) and often found in biofilms of trickle-bed reactors (Porté *et al.* 2019).

The role of the inoculum was investigated in a batch experiment by daily injection of H_2 to the headspace, maintaining a 1 bar partial pressure over a 12 days period (Braga Nan *et al.* 2020). Wide variations of product spectra were observed and besides the main products of acetate and methane, other VFAs were detected including propionate, butyrate, isobutyrate, and isovalerate. Most effective biomethanation was observed when inocula dominated by hydrogenotrophic methanogens and the versatile *Methanosarcina* was also present. The role of the inoculum microbial diversity was also highlighted by Logroño et al. (Logroño *et al.* 2022) showing that highly diverse cultures, including acetoclastic methanogens, besides the predominant hydrogenotrophic ones outperformed the medium and low diversity cultures in the long-term operation of *ex situ* biomethanation. Tsapekos et al. (Tsapekos *et al.* 2022) found that an inoculum adapted to hydrogen utilization was able to convert H_2/CO_2 mixtures at various initial pressures to methane, while acetate accumulation and predominance of homoacetogenesis was observed in the case of a non-adapted inoculum.

Most studies, thus far, have focused on characterizing the methanogenic communities. However, there is huge potential in isolating strains from complex communities that are adapted to cope with disturbances that are relevant for practical implementation, like starvation or change in temperature and pH.

Microbial electrochemical methanation

Microbial electrochemical methanation (also called bioelectromethanation or electromethanogenesis) is the combined electrochemical and biological methane production in one bioelectrochemical system. Methanogens use CO_2 and electrons directly provided by a cathode and protons from the liquid phase (Fu *et al.* 2021) or *in situ* produced hydrogen, as well as other electron carriers, such as formate, that have been produced at the cathode (Kracke *et al.* 2019). Thereby, electroactive microorganisms used for bioelectromethanation perform either direct or indirect (mediated) extracellular electron transfer (EET) (Schröder *et al.* 2015).

Direct EET requires specific structures, such as membrane-bound c-type cytochromes, to transfer electrons. In electroactive bacteria performing direct EET, *e.g.*, the archetype *Geobacter* sp., type IV pili or protein nanowires comprised of the c-type cytochrome OmcS (Lovley and Holmes 2020) facilitate electron transfer over longer distances in biofilms where the microorganisms have no immediate contact to the electrode. This allows them to form anodic biofilms with a thickness of more than 100 µm (Virdis *et al.* 2014; Baudler *et al.* 2015; Sun *et al.* 2015; Blanchet *et al.* 2016; Dhar *et al.* 2017). In archaea, these e-pili are called archaella and are homologous to type IV pili of bacteria (Lovley and Holmes 2020).

Indirect or mediated EET means that a mediator is required to shuttle electrons between the microorganisms and the electrode. At anodes, these are usually redox molecules, such as flavins, phenazines or quinones (Patil *et al.* 2014; Schröder *et al.* 2015; Saunders and Newman 2018; Yee *et al.* 2020). For mediated EET at cathodes, the mediator needs to be transported to the methanogens to be taken up and oxidized. The simplest form of such a mediator is electrochemically produced hydrogen, utilized by hydrogenotrophic methanogens to produce methane (see Eq. 1). A further but much less discussed opportunity is hydrogen production at

cathodes catalyzed by microbial extracellular hydrogenases or formate synthetases that catalyze hydrogen or formate production by reducing the overpotential of the reaction (Deutzmann *et al.* 2015). Instead of biological catalysts, optimization of electrode material for abiotic *in situ* production of hydrogen is an alternative approach (Kracke *et al.* 2019, 2020).

Due to the manifold opportunities of electron transfer and the property of hydrogenotrophic archaea to keep the hydrogen partial pressure extremely low, it is very difficult to differentiate between direct and indirect EET during microbial electrochemical methanation. Consequently, detecting no hydrogen in microbial electrochemical methanation systems (both in the liquid and gas phase) is not a sufficient argument to conclude that direct EET is taking place.

As mentioned earlier, electroactive bacteria performing direct EET rely on electron transfer structures, such as c-type cytochromes. In the case of methanogenic archaea, the situation seems to be somewhat more complex, as there exist methanogens with multi-heme cytochromes *e.g.*, *Methanosarcina mazei* or *Methanosarcina barkeri*, but also methanogens without cytochromes, e.g., Methanobacteriales, Methanococcales, Methanomicrobiales and Methanopyrales (Thauer *et al.* 2008). The first study claiming direct electron uptake by a cytochrome-free methanogenic archaeon showed that methane was formed by a hydrogenase-independent mutant strain (*Methanococcus maripaludis* MM1284), but the evidence was not conclusive (Lohner *et al.* 2014). Alternatively, bioelectromethanation with a defined co-culture of the Fe(0)-corroding strain IS4 (hydrogen producer) and *Methanococcus maripaludis* (H_2 consumer) showed a 20 times higher MPR than using a single strain (Deutzmann and Spormann 2017). Other methanogens, namely *Methanolacinia petrolearia, Methanobacterium congolense* and *Methanoculleus submarinus* have also been tested for bioelectromethanation at -700 mV (vs. standard hydrogen electrode (SHE)), a potential where abiotic H_2 formation at

graphite electrodes is unlikely (Mayer *et al.* 2019). Recently, the intrinsic electroactivity with a cathode poised at -400 mV (vs. SHE) was demonstrated for *Methanosarcina barkeri* strain MS because of its ability to perform bioelectromethanation but not for *Methanosarcina horonobensis* (Yee *et al.* 2019). Likewise, *Methanosarcina mazei* (wild type and multiheme c-type cytochromes knockout mutant) was shown to be capable to take up electrons from a cathode and the study suggested that multi-heme c-type cytochromes were not required for electron uptake (Yee and Rotaru 2020).

A study with cytochrome-containing methanogens (*Methanosarcina barkeri*) found that methane was formed after several days of incubation when the cathode was poised at −400 mV (vs. SHE) in a microbial media as sole electron donor (Yee *et al.* 2019; Yee and Rotaru 2020). However, the MPR can increase when H_2 is available (Deutzmann and Spormann 2017), thus a H_2-dependent microbial electrochemical methanation system may be advantageous. Recently, *Methanococcus maripaludis* and *Sporomusa ovata* were used in a H_2-generating bioelectrochemical reactor to support the microbial CO_2 reduction to CH_4 and acetate, respectively (Kracke *et al.* 2019). The study stands out because of the use of non-precious metal cathodes (CoP, MoS_2 and NiMo) and coulombic efficiencies close to 100% without accumulating hydrogen. In a follow-up study, the authors achieved an unprecedented MPR of 1.4 L^{-1} d^{-1} when using *Methanococcus maripaludis* and NiMo cathodes (Kracke *et al.* 2020). From the application point of view, the authors demonstrated an *in situ* H_2-producing reactor with high MPR and robust growth of microbial biomass. The methanogens in this reactor showed a proteomic pattern similar to that observed in reactors fed with a H_2/CO_2 gas mixture. Moreover, since the cathode was made of an earth-abundant material, it opens a window for further application (Kracke *et al.* 2020). Although *M. maripaludis* is a model organism and genetic tools

are available, other methanogenic strains and especially thermophilic strains depicting higher MPR (Rittmann *et al.* 2015) should be tested in the system developed by Kracke *et al.* (2020). As recent studies have shown excellent potential of thermophilic and hyperthermophilic methanogenic strains for successful process development (Mauerhofer *et al.* 2018, 2021), further research should explore these strains for microbial electrochemical methanation.

Mixed cultures have also been used to perform microbial electrochemical methanation (Molenaar *et al.* 2017; Yang *et al.* 2018, 2020; Zhou *et al.* 2020). A major drawback of using mixed cultures is the formation of by-products, such as acetate or propionate (Yang *et al.* 2020), although this seems to be decreased under thermophilic compared to mesophilic conditions (Yang *et al.* 2018). Since thermophilic methanogens show a higher MPR than mesophilic ones, biomethanation of flue gas without cooling or biogas upgrading should be tested. Implementation of bioelectrochemistry into an AD reactor design is an alternative way of *in situ* biogas upgrading. A single-chamber stainless steel reactor combining a microbial electrolysis cell and AD was developed by Bo *et al.* (2014). The inner surface of a stainless steel reactor served as cathode through small voltage addition (1.0 V) and generated H_2, while a carbon felt served as anode. In comparison to a reference reactor, the CH_4 yield doubled, resulting in 98% CH_4 content in the biogas, while the COD removal rate was increased three times (Bo *et al.* 2014). Biogas upgrading was demonstrated in the cathode compartment of a membraneless microbial electrosynthesis cell, which significantly reduced the required biogas retention time as well as energy consumption for biogas upgrading compared to injection of H_2 through sparging or a biofilter approach (Tartakovsky *et al.* 2021). A high efficiency *in situ* biogas upgrading bioelectrochemical system with low energy input for the treatment of artificial wastewater with acetate was developed by Liu *et al.* (2021). The authors attributed the high efficiency to a

significant enrichment of *Methanothrix* on the cathode surface, which expressed genes involved in acetoclastic methanogenesis and direct electron transfer. Increased proton consumption caused a higher pH and hence higher solubility of CO_2 in the bioreactor, which resulted in a methane content of 97% in the gas phase (Liu *et al.* 2021).

The genus *Methanobacterium* appears to occupy a relevant niche in biomethanation of H_2 or microbial electrochemical methanation with mixed cultures (Yang *et al.* 2018). Therefore, the combination of H_2 production under biocompatible conditions with hydrogenotrophic methanogenesis that avoids by-products (Kracke *et al.* 2020) came timely to push forward this technology.

POWER-TO-CHEMICALS

Bioelectrochemical production of chemicals from CO_2 and electricity is promising, because point sources of CO_2 emission can be captured and surplus electricity can be stored. This is feasible using immediate "feeding" of electrons to microorganisms in primary microbial electrochemical technologies (MET) (Rabaey and Rozendal 2010; Jourdin *et al.* 2015; Vassilev *et al.* 2018; Jourdin and Burdyny 2021). Primary MET can be based on mediated or direct EET as introduced earlier (Schröder *et al.* 2015). A number of reviews cover MET for synthesis of chemicals based on direct ETT (Nevin *et al.* 2010; Schröder *et al*. 2015; Dessì *et al.* 2021; Jourdin and Burdyny 2021; Fang *et al.* 2022), which usually results in mixtures of short- to medium-chain carboxylic acids (acetic, butyric, and caproic acid) from CO_2 and electric energy. Here, we focus on the abiotic electrochemical reduction of CO_2 to the C1 compound formate/formic acid that is used for biosynthesis either *in situ* or subsequently (Izadi and Harnisch 2022).

The abiotic electrochemical reduction of CO_2 to formate was first reported by Li *et al.* (2012) and later demonstrated under bio-compatible conditions in a bioreactor at laboratory scale (Hegner *et al.* 2019). Since microbial CO_2 fixation is relatively slow (Tashiro *et al.* 2018), an electrochemically produced intermediate, such as formate, is generated to feed microorganisms that produce the chemicals of interest. Studies combining electrochemical and microbial reactions for the production of chemicals of interest have almost exclusively used engineered strains as biocatalysts. A pioneering study demonstrated the reduction of CO_2 to formate and its subsequent utilization to produce higher alcohols, such as isobutanol and 3-methyl-1-butanol, by the genetically engineered aerobic bacterium *Cupriavidus necator* H16 (formerly called *Ralstonia eutropha*) as the production host (Li *et al.* 2012). Recently, a proof of concept study demonstrated CO_2 reduction to formate with a copper catalyst and the subsequent microbial conversion of formate to acetate and methane (Chatzipanagiotou *et al.* 2020). It has also been demonstrated that the bioplastics precursor poly(3-hydroxybutyrate) (PHB) was produced by *Cupriavidus necator* H16 from formate derived from electrochemical CO_2 reduction in a single bioreactor (Salem *et al.* 2018) and in a two-stage process (Stöckl *et al.* 2020). The first evidence of autotrophic terpene production from CO_2, H_2, and O_2 was devised when electrochemically produced H_2 was used to feed an engineered *Cupriavidus necator* strain (PHB-deficient strain) to produce the terpene *α*-humulene (Krieg *et al.* 2018).

However, apart from the electrochemical target reaction of CO_2 reduction, also side reactions need to be considered. At the cathode these are the hydrogen evolution as well as the oxygen reduction reaction. Since electrochemical reduction of oxygen can produce bacterial stressors, such as hydrogen peroxide, peroxide free radicals or via further radical reactions nitric oxide (NO) (Li *et al.* 2012), an anaerobic system is desirable. Furthermore, it remains to be clarified, if

integrated production in electro-bioreactors (Rosa *et al.* 2019) or rather a production cascade using two-step processes is more favorable in the long term (Abel and Clark 2021). *Escherichia coli*, a facultatively anaerobic bacterium, was engineered to produce pyruvate from CO_2 and formate in a single reactor wherein formate was electrochemically produced. Such a system could facilitate the production of a wide variety of chemicals by integrating almost any biochemical pathway (Tashiro *et al.* 2018; Claassens *et al.* 2019; Cotton *et al.* 2020; Kim *et al.* 2020). A recent study showed that polymer bricks, such as mesaconate and 2S-methylsuccinate, can be produced with such hybrid systems by using the genetically engineered strain *Methylobacterium extorquens* AM-1 (Hegner *et al.* 2020). Furthermore, 1-butanol and 1-hexanol were produced from CO_2 and H_2O, whereupon a CO_2 electrolyzer provided syngas (CO) to feed an acetogenic microbial consortium composed of *Clostridium autoethanogenum* and *Clostridium kluyveri* (Haas *et al.* 2018). This CO route is of similar interest as the route via formate, especially as formaldehyde is most toxic among the C1 compounds and methanol is most challenging from an electrochemical perspective (Stöckl *et al.* 2022).

POWER-TO-PROTEIN

For millennia, humankind has relied on animal products as their prime source of protein. In the last decades, both ethical and ecological concerns have paved the road towards soybean or other plant-based sources of protein. However, even the production of soybean is unsustainable, due to massive deforestation and loss of biodiversity, dependency on import in the EU and China, net GHG emissions and huge nitrogen losses (Castanheira and Freire 2013; Pikaar *et al.* 2017). Even though novel efforts resulted in an uncoupling of deforestation and soybean production (Macedo

et al. 2012), the current annual yield of 3-4 tons per hectare (Matassa *et al.* 2015; Martinelli *et al.* 2017; Pikaar *et al.* 2017) is not sufficient to keep on sustaining the growing world population. This requires the need for alternative protein sources for which microbial protein (MP) is an example (De Vrieze *et al.* 2020b; Marcellin *et al.* 2022).

MP can (i) reach a productivity of up to annual yield of 3000 tons per hectare, which is a factor 1000 higher than soybean, (ii) achieve carbon neutrality or at least strongly reduce carbon emissions, and (iii) obtain high nitrogen use efficiencies of 43%, in contrast to 14% for soybean (Matassa *et al.* 2016a; Pikaar *et al.* 2017, 2018). MP has a complete profile of valuable amino acids and the total essential amino acids content of certain strains is more than 10% higher than in wheat grain (Volova and Barashkov 2010). The future success of MP as third-generation protein will depend on the versatility and robustness of the key microorganisms involved, and their ability to efficiently employ renewable energy sources. Here, we discuss the potential of employing electric power to produce MP, for which three different routes using hydrogen, methane as energy source are considered (Fig. 2). The current status and future perspectives concerning the involved microorganisms and their potentials are evaluated.

The hydrogen route: hydrogen-oxidizing bacteria

The cost of the production of renewable electricity, *i.e.*, from solar or wind energy, and further conversion to hydrogen by water electrolysis has been decreasing over the last years. This has intensified research efforts to produce MP using the hydrogen route through hydrogen-oxidizing bacteria (HOB) or Knallgas bacteria (Matassa *et al.* 2015). Already in the 1960s, the application of HOB was considered as a way to control the atmosphere of a space cabin and to provide nourishment for astronauts during longer space missions (Foster and Litchfield 1964; Waslien *et al.* 1969). In the 1970s, HOB were used for the production of MP using a pure culture of

Cupriavidus necator (formerly named as *Alcaligenes eutrophus*) (Repaske and Mayer 1976). Renewed interest in the 2010s resulted in a multitude of studies using either pure or enrichment cultures to produce MP using the hydrogen gas route (**Table 2**). The HOB comprise a heterogeneous group with representatives in several genera, including the Gram-negatives *Pseudomonas*, *Aquaspirillum*, and *Flavobacterium*, and the Gram-positives *Nocardia*, *Mycobacterium, Corynebacterium*, and *Bacillus* (Schink and Schlegel 1978), which enlightens their apparent broad potential for engineering applications.

Key advantages of HOB with respect to MP production are (i) their ability to use CO_2 as carbon source, even though they can switch to organic carbon sources as well (Pohlmann *et al.* 2006; Dou *et al.* 2019), (ii) their high biomass productivity of 0.38 g cell dry weight (CDW) $L^{-1}h^{-1}$ and biomass yields of 2.2-2.4 g CDW g^{-1} H_2 (Matassa *et al.* 2016b), and (iii) their ability to use recovered nutrients, as demonstrated for ammonia recovered from urine (Christiaens *et al.* 2017). HOB can use different nitrogen sources, *i.e.*, ammonia (Matassa *et al.* 2016b) and nitrate (Zhang *et al.* 2020), and several strains can even fix N_2 as nitrogen source (Hu *et al.* 2020). Similar to other P2X applications, an important disadvantage is the low solubility of H_2 at ambient temperature and pressure, either requiring the need for high-pressure conditions or specific gas supply systems and reactor configuration to sustain efficient H_2 supply and related high MP production rates. In addition, the high nucleic acid content of bacterial biomass, between 8-16 % of the dry weight (Anupama and Ravindra 2000; Strong *et al.* 2015; Clauwaert *et al.* 2017), could have a negative impact on the health of the consumer (animals or humans), and this requires an additional treatment step (Sillman *et al.* 2019).

Overall, their high diversity and versatility make HOB excellent candidates for full-scale MP production from hydrogen. This fits within the Power-to-Protein concept (Mishra *et al.* 2020) and is currently being evaluated at the pilot scale by the companies Avecom nv (Ghent, Belgium) and Solar Foods (Lappeenranta, Finland).

The methane-methanol route: methanotrophic and methylotrophic bacteria

MP production from methane reflects the second indirect route of utilizing electric power. The decreasing cost of H_2 production, as mentioned earlier, not only attracted interest in the hydrogen route for MP production but also created the potential for *in situ* or *ex situ* upgrading of biogas from AD processes to biomethane within the P2G concept (Collet *et al.* 2017; Angelidaki *et al.* 2018). Methanotrophic or methane-oxidizing bacteria (MOB) represent a heterogeneous group of microbes with bacterial representatives in the families Methylococcaceae, Methylocystaceae, Methylothermaceae and Beijerinckiaceae (phylum Proteobacteria), Methylacidiphilaceae (phylum Verrucomicrobia), in the NC10 phylum, and several more candidate species (Dedysh and Knief 2018). Methanotrophic bacteria convert methane to CO_2 and biomass, using either oxygen, nitrate, nitrite, manganese, iron or sulfate as terminal electron acceptor (Op den Camp *et al.* 2018). From a MP production point of view, the aerobic route, *i.e.*, using O_2 as electron acceptor, can be considered the most promising because of the highest biomass yields and production rates compared to other electron acceptors (van Grinsven *et al.* 2020).

There is some progress in electrochemical oxidation of methane to methanol (Fornaciari *et al.* 2020; Jiang *et al.* 2022) and in selective electroreduction of carbon dioxide to methanol (Yang *et al.* 2019), which is a very attractive way to provide a water-soluble reduced C1 compound that can be also used for MP production. The onset of MP production from methanol, already at the full scale, dates back to the production of MP under the trade name “Pruteen” from natural gas

by Imperial Chemical Industries Ltd in the 1960s and 1970s, using a *Methylophilus methylotrophus* strain (Braude *et al.* 1977). A renewed interest in the 2000s and 2010s resulted in the production of MP through the methanotrophic routes by, amongst others, the companies Calysta (FeedKind® Protein, Menlo Park, CA) and Unibio A/S (UniProtein®, Lyngby, Denmark), both using a *Methylococcus capsulatus* strain (Strong *et al.* 2015). At present, mainly *Methylococcus capsulatus* is used at the full- and lab scale for MP production from methane, often supported by heterotrophic "assisting" bacteria (Bothe *et al.* 2002).

Key advantages of MOB with respect to MP production are (i) their ability to directly use natural gas, biomethane or even raw biogas (Khoshnevisan *et al.* 2019), (ii) despite their low biomass productivity of 0.04 g CDW $L^{-1}h^{-1}$, yet high biomass yields of 0.80 g CDW g^{-1} CH_4 (Matassa *et al.* 2015), which can be partially compensated by much higher solubility of CH_4 (23 mg L^{-1} at ambient temperature and pressure) compared to H_2 (1.6 mg L^{-1}). Another advantage is (iii) their ability to use recovered nutrients as demonstrated by the direct growth of methanotrophs in pasteurized centrifuged-filtered digestate (Khoshnevisan *et al.* 2019) or using electrochemically extracted ammonia nitrogen (NH_4^+) from digested biowaste (Tsapekos *et al.* 2020). Another key advantage from an engineering point of view is the possibility to achieve MP production from growing MOBs together with microalgae, as first demonstrated in the "methalgae" concept (van der Ha *et al.* 2011), and now being applied for MP production (Rasouli *et al.* 2018). Even co-culturing/enriching of HOB and MOB together to achieve efficient MP production from gas mixtures is a possible approach (Acosta *et al.* 2020). An important disadvantage resides in the fact that biomass from MOB, similar to HOB, have a high nucleic acid content (Strong, Xie and Clarke 2015). Moreover, the potential release of non-utilized methane, which has a global

warming potential of 28 CO_2 equivalents (Saunois *et al.* 2016), should be avoided during production of MP, especially given the higher solubility of CH_4 compared to H_2.

Overall, the production of MP from methane, at present, seems to have reached a higher TRL (technology readiness level) than the hydrogen route. The direct usage of biogas or biomethane rather than natural gas can be an important asset to achieve energy-efficient production of MP in combination with organic waste valorization through AD.

The acetate route: acetate-utilizing microbes

Bioelectrochemical or homoacetogenic acetate production is a mature technology that could be used for the production of MP in a two-step process. Independent of the production approach, acetate can be used as feedstock for the production of protein for feed or food applications. Such a system was developed in which acetate is produced by the acetogen *Clostridium ljungdahlii,* thereby fixing carbon dioxide using renewable hydrogen as electron donor and subsequently feeding acetate to *Saccharomyces cerevisiae* for the production of microbial protein (Molitor *et al*. 2019). A carbon yield of up to 25% of yeast biomass with a protein fraction of 40-50% has already been achieved in this proof-of-concept study. This is a sound approach to produce MP and circumvent regulatory hurdles, since *S. cerevisiae* has a long history of use in human nutrition and has GRAS (Generally Recognized As Safe) status. Alternatively, it is also possible to perform electrochemical reduction of carbon dioxide to fix carbon and produce oxygen and acetate (other potential products also include formate, methanol or methane) to then feed heterotrophic microbes and produce MP (Mishra *et al.* 2020). Acetate could also be used to feed filamentous fungi like *Aspergillus oryzae* DSM 1863, which is capable of using acetate as carbon source as highlighted recently by Kövilein *et al*. 2021). Although the study originally focused on

L-malic acid production, biomass production could also be possible. The comparison of formate and acetate as substrate for MP production was performed by pure cultures and enrichment cultures (Sakarika *et al.* 2020). The performance was characterized kinetically, stoichiometrically and nutritionally and they found that growth on acetate was better and the protein content was the highest during stationary phase.

Using two-step bioprocesses leads to an increased complexity, but it could be an elegant way to produce MP while fixing carbon dioxide and using surplus electricity from renewable sources.

In conclusion, each of the routes has its opportunities, but also reflects specific challenges. While the production of MP through MOB has applications at the full scale, the utilization of HOB offers new opportunities in the context of integrated resource recovery. The vast potential of unexplored microbial diversity, both pure and mixed cultures, is there, and waits to be applied in our efforts to substantiate the new generation of MP. However, one should not neglect key aspects of food safety and potential refusal by consumers when the application of MP for feed production is to be extended towards food production. Isolation of new strains combined with genome analysis should be used in future to exclude those microorganisms potentially producing toxins and allergens. Another main challenge is to simulate the desirable appearance, texture, flavor, and functionality of food products using ingredients that are isolated from microbial sources.

APPLICATION OF MOLECULAR METHODS FOR THE ANALYSIS OF REACTOR MICROBIOMES IN P2X SYSTEMS

Microbial community analysis: the basics

In addition to providing knowledge on the taxonomic composition and even functional traits as well as dynamics of microbial communities, modern molecular techniques can be used for direct process monitoring (Fig. 3). Thereby molecular markers may allow process steering and provide pre-warning signals of process failure (Lv *et al.* 2014; Leite *et al.* 2016; De Vrieze *et al.* 2018; Lambrecht *et al.* 2019).

The standard approach of cultivation-independent analysis of complex microbial communities involves the isolation of nucleic acids from the samples, followed by polymerase chain reaction (PCR) amplification of a phylogenetic marker gene, which is in most cases the 16S rRNA gene. Due to the phylogenetic diversity of microorganisms, the design of universal primers perfectly matching all target genes cannot be achieved. This leads to preferential amplification of certain taxa and hence a certain bias (Sipos *et al.* 2007). Nevertheless, the frequently used domain-specific primers can give a broad overview of the microbial diversity and can be used to follow community dynamics (Klindworth *et al.* 2013). Using archaea-specific 16S rRNA gene primers, Guneratnam *et al.* (2017) investigated the methanogenic community in a thermophilic *ex situ* biomethanation process by cloning and Sanger sequencing of the PCR products. The most abundant clones were affiliated to *Methanothermobacter wolfei* and *Methanothermobacter thermautotrophicus*.

Before the advent of high-throughput sequencing techniques, molecular fingerprinting methods had been developed for the fast comparison of PCR products from numerous samples by providing profiles or patterns describing the diversity of amplified DNA sequences and enabling the study of temporal shifts and spatial heterogeneities in microbial community structure (De Vrieze *et al.* 2018). Denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA amplicons are today considered outdated techniques. However, DGGE was a highly valuable tool in the past, e.g. to characterize the methanogenic communities in a bioelectrochemical system during electromethanogenesis. The biocathode in this system was dominated by a single archaeon, *Methanobacterium palustre*, while less abundant community members were affiliated to *Methanoregula boonei* and *Methanospirillum hungatei* (Cheng *et al.* 2009). Luo and Angelidaki also used DGGE to identify the most abundant methanogens in an *ex situ* thermophilic biomethanation and found that addition of hydrogen enriched methanogens of the order Methanobacteriales (Luo and Angelidaki 2012). *In situ* biogas upgrading of thermophilic co-digestion of manure and whey also resulted in the increase of *Methanothermobacter thermautotrophicus* (order Methanobacteriales) according to the assessment by DGGE (Luo and Angelidaki 2013b). Addition of taxonomic information to the DGGE community patterns is possible, but direct sequencing of DNA recovered from cut out bands might result in mixed sequences, and more precise analysis requires the establishment of supporting clone libraries (Nikolausz *et al.* 2005).

High-throughput microbial community analysis by cytometry

Flow cytometry can be applied to investigate optical characteristics of individual cells of complex microbial communities in high throughput (Koch *et al.* 2014); therefore, it is a

promising method for monitoring the dynamics of P2X reactor microbiomes. By measuring light scatter behavior, which is related to cell size and granularity, as well as light signals resulting from autofluorescence, e.g., the F_{420} autofluorescence of methanogens (Lambrecht *et al.* 2017), or fluorescent dyes staining the DNA or other cell constituents, the recorded data are visualized in cytometric histograms. Such cytometric fingerprinting can be used to compare the structure and dynamics of the reactor microbiota in combination with the routine assessment of process parameters (Koch *et al.* 2014). It has been used for the analysis of wastewater treatment plants (Günther *et al.* 2012, 2016; Vučić *et al*. 2021) or AD systems (Koch *et al.* 2013, Lambrecht *et al.* 2017, Günther *et al.* 2018), and shows a strong relation with other high-throughput techniques, at least at the *β*-diversity level (De Vrieze *et al.* 2021).

An interesting combination of AD and microbial electrolysis cells was investigated by cytometric fingerprinting and T-RFLP profiling targeting both 16S rRNA and *mcrA* genes (Koch *et al.* 2015). A 27% increase in total gas yield was achieved by bioelectrochemical enhancement compared to standard AD without affecting the major community dynamics in the bulk liquid. However, specific enrichments of *Geobacter* sp. on the anode and *Methanobacterium* sp. on the cathode were observed (Koch *et al.* 2015). Flow cytometry based cell sorting can increase the resolution of other molecular methods and omics methods as individual sub-communities can be subjected to amplicon sequencing, metagenome and metaproteome analyses (Abdulkadir *et al*. 2023).

The 16S rRNA gene as phylogenetic marker

Next generation high-throughput sequencing technologies address the challenge of low taxonomic resolution of molecular fingerprinting methods by providing an unparalleled resolution of the diversity (sequencing depth) at reduced cost and time. Amplicon sequencing of

the 16S rRNA genes by employing sequencing platforms such as Illumina is the standard approach for microbial community analysis nowadays. It was used, for example, to investigate the inoculation of a biotrickling filter for hydrogenotrophic methanogenesis. After stable operation, the genera *Methanobacterium* and *Methanothermobacter* together represented more than 93% of the archaeal sequences (Dahl Jønson *et al.* 2020). The effect of process temperature on the microbial community involved in hydrogen biomethanation showed that *Methanobacterium* was predominant at lower temperatures, while *Methanothermobacter* became more abundant at higher temperatures. Among the bacteria, the putative syntrophic acetate-oxidizing genera *Coprothermobacter* and *Caldanaerobacter* were found to be predominant (Xu *et al.* 2020). The temperature dependence of bioelectrochemical CO_2 conversion and methane production was investigated during microbial electrochemical methanation with a mixed-culture biocathode, and a decrease in the relative abundance of *Methanothrix* with increasing temperature was observed, while *Methanobacterium* became the predominant archaeon (Yang *et al.* 2018). The active fraction of the microbial community, analyzed on the rRNA level, of a primary MET generating acetate and methane from CO_2 as sole carbon source pointed at *Methanobacterium* spp. and *Acetobacterium* spp. as the most abundant active archaea and bacteria, respectively (Marshall *et al.* 2012). Bioelectrochemically enhanced AD in a UASB reactor to improve the CH_4 production and organic matter removal at a short hydraulic retention time resulted in enhanced relative abundance of the genera *Methanobrevibacter*, *Bifidobacterium*, *Clostridium*, *Pectinatus* and *Megasphaera* (Li *et al.* 2016). In another study, the relative abundance of hydrogenotrophic methanogens, such as *Methanothermobacter thermautotrophicus*, increased as a result of H_2 addition, together with syntrophic bacteria of the genera *Anaerobaculum, Thermacetogenium, Tepidanaerobacter, Syntrophomonas* (Bassani *et al.*

2017). A similar approach was used by the same group analyzing various thermophilic reactors and reactor configurations, *i.e.*, CSTR, serial upflow and bubble column reactors. Operational taxonomic units (OTUs) belonging to the orders MBA08 (class Clostridia) and Bacteroidales were found as most abundant bacteria, and *Methanothermobacter thermautotrophicus* was the most abundant methanogen followed by the less abundant (below 2%) *Methanoculleus palmolei* (Kougias *et al.* 2017). H_2 addition for *in situ* biogas upgrading (Mulat *et al.* 2017) resulted in an enrichment of hydrogenotrophic *Methanobacterium* and indicated the key role of this genus and hydrogenotrophic methanogenesis to increase the CH_4 concentration up to 89%, as confirmed through microbial community and carbon stable isotope analysis of CH_4 and CO_2. Excess H_2 addition resulted in accumulation of H_2, depletion of CO_2 and inhibition of VFA degradation.

Only few studies analyzed the microbial community composition in case of MP production with mixed cultures. Matassa and co-workers established various enrichment cultures and operated reactors under various conditions for evaluating the potential of HOB to upgrade ammonium and carbon dioxide under autotrophic growth using aerobic sludge from a local food processing plant as inoculum (Matassa *et al.* 2016b; Christiaens *et al.* 2017). The genera *Ancylobacter*, *Xanthobacter* (Alphaproteobacteria) and *Hydrogenophaga* (Betaproteobacteria) were the most abundant in sequencing batch reactor mode. An interesting finding was that one third of the sequences were affiliated to the genus *Bdellovibrio* (Deltaproteobacteria), including mainly predatory bacteria probably feeding on the autotrophic HOB. The remaining detected genera were mainly affiliated to the classes Flavobacteriia and Sphingobacteria. In continuous feeding mode, 97% of the sequences were affiliated to a single genus, *Sulfuricurvum* (Epsilonproteobacteria) (Matassa *et al.* 2016b). A similar approach was used to enrich novel HOB from high-temperature and high-salinity environments. While the genus *Achromobacter*

was found to dominate saline enrichments, the genus *Hydrogenibacillus* was found to be predominant in thermophilic enrichments. The thermophilic enrichments had the highest protein content, and increased temperature was assumed to be advantageous against infection of the cultures with pathogens (Barbosa *et al.* 2021).

Long-term microbiological surveys of large numbers of reactor samples with standard methods are necessary to provide comprehensive datasets for the deeper understanding of the characteristics and links between key variables and the microbial community composition. A good example is the study by Jiang *et al*. (2021), which analyzed 46 anaerobic digesters at Danish wastewater treatment plants over six years by amplicon sequencing of the 16S rRNA genes and using an ecosystem-specific reference database (MiDAS 3) and species-level identification using amplicon sequencing variants (ASV) instead of the outdated OTU approach at 97% sequence similarity. Such long-term monitoring of large numbers of reactors with microbiota should also be done for P2X systems. It could help elucidate the potential ecological function of so far uncharacterized taxa and relationships between specific taxa and key parameters, hence enabling potential improvement of process performance.

Functional marker genes

The analysis of functional marker genes instead of 16S rRNA genes is an alternative approach that allows the targeted investigation of distinct functional guilds. The *mcrA* gene encoding the alpha-subunit of methyl coenzyme M reductase is ubiquitous but specific for methanogens and it is the most widely used molecular marker for the assessment of biomethanation processes (Lueders *et al.* 2001; Luton *et al.* 2002). Another advantage of the *mcrA* gene is that its transcripts can provide information about the active members of methanogenic communities

(Munk *et al.* 2012; Nikolausz *et al.* 2013; Lv *et al.* 2014; Wintsche *et al.* 2016). Due to the relatively low diversity of methanogens, it was possible to develop a T-RFLP approach based on an improved primer set (Steinberg and Regan 2008) and a database facilitating the fast identification of methanogens, thus avoiding the need of cloning and sequencing (Bühligen *et al.* 2016). Ács and colleagues combined the T-RFLP analysis of *mcrA* genes and Ion Torrent whole metagenome DNA sequencing to investigate the effect of substrate shift from cellulose to H_2 and CO_2 on the microbiota in simple fed-batch cultures. The abundance of several genera, in particular *Candidatus Cloacimonas* and *Herbinix*, was observed to increase during H_2 feeding. The genus *Methanobacterium* represented the most abundant methanogen in every reactor, but *Methanoculleus* also benefitted from substrate change, while *Methanothrix* persisted (Ács *et al.* 2019). Agneessens and co-workers found an increase in relative abundance of an OTU affiliated to the Methanobacteriales from below 1% to 6.1% after H_2 addition to a system digesting sludge and straw by the application of *mcrA* gene amplicon sequencing (Agneessens *et al.* 2017).

Logroño and co-workers combined the amplicon sequencing of 16S rRNA and *mcrA* genes to assess the community structure of bacteria and methanogenic archaea in *ex situ* mesophilic biomethanation enrichment systems, feeding H_2 and CO_2 at alkaline pH (Logroño *et al.* 2020). The bacterial community was dominated by a member of the genus *Lutispora* that was suspected to contribute to homoacetogenesis. The predominant methanogens belonged to the genera *Methanobacterium* and *Methanoculleus*. The same molecular approach was used to investigate the resilience of the biomethanation community upon starvation in mesophilic fed-batch reactors inoculated from two different sources. Both communities showed functional resilience for starvation periods of up to 14 days. The predominance of the hydrogenotrophic methanogen

Methanobacterium in the inoculum was suggested to be important for an efficient and resilient process (Logroño *et al.* 2021).

Microorganisms involved in reductive acetogenesis or syntrophic acetate oxidation employing the Wood-Ljungdahl pathway can be targeted by the *fhs* gene encoding the formyltetrahydrofolate synthetase (FTHFS) (Leaphart and Lovell 2001; Lovell and Leaphart 2005; Henderson *et al.* 2010). However, this molecular marker is not specific for homoacetogens since the FTHFS is also involved in other pathways (Gagen *et al.* 2010; Westerholm *et al.* 2011; Hädrich *et al.* 2012). Therefore, it has been suggested to use the acetyl-CoA synthase (ACS) as functional marker for the Wood-Ljungdahl pathway, and primers targeting its gene *acsB* have been designed (Gagen *et al.* 2010). Compared to the methanogens targeted by the *mcrA* gene, the functional diversity of homoacetogens in H_2 biomethanation systems is not yet well explored but the *fhs* marker has been frequently used to detect potential homoacetogens or syntrophic acetate-oxidizing bacteria (SAOB) in AD. For instance, Westerholm *et al.* 2011 studied the *fhs* gene diversity in a mesophilic laboratory-scale biogas reactor, the *fhs* gene abundance in natural and engineered environments was analyzed by qPCR (Xu *et al.* 2009), and homoacetogenic activity during acidification in a thermophilic AD system was investigated targeting the *fhs* gene (Akuzawa *et al.* 2011). More recently, *fhs* gene amplicon sequencing was used in microbiological surveillance of biogas plants (Singh *et al.* 2020, 2021a; Singh *et al.* 2021b), and the *fhs* gene database AcetoBase was established (Singh *et al.* 2019; Singh and Schnürer 2022). In the study of Braga Nan *et al.* (2020), quantification of the FTHFS gene was performed by using qPCR to assess homoacetogenesis in batch cultures with seven different inocula fed with hydrogen but no clear correlation between acetic acid accumulation and copy number of the target gene was observed.

The "omics" approach

The advances in sequencing technologies made it possible to analyze not only single genes, but to target all genes or transcripts even in complex microbial communities. Metagenomics is a complex approach including high-throughput sequencing and bioinformatics tools to characterize the entire genetic content of microbial communities without a preceding PCR step (Thomas *et al.* 2012). In a similar manner, metatranscriptomics is defined as the approach to characterize the expressed genes of a community by deep sequencing the reverse-transcribed RNA isolated from a complex sample (Fig. 3).

One strategy is to directly analyze the unassembled sequence data (gene-centric metagenomics) by comparing reads directly to protein databases without linking metabolic functions to specific organisms (Jaenicke *et al.* 2011; Wirth *et al.* 2012; Li *et al.* 2013). A more ambitious goal of metagenomics is the complete coverage of all genes and the re-construction of whole genomes of interacting populations (genome-centric metagenomics), which allows to link functional and taxonomic information. Genome-centric metagenomics was applied to biogas upgrading reactors (*in situ* biomethanation), both at mesophilic and thermophilic conditions, and revealed the predominance of two closely related *Methanoculleus* spp. possessing different metabolic features depending on the temperature. Previously not yet characterized syntrophic bacteria with potential homoacetogenic activity were also described (Treu *et al.* 2018). A metagenomics approach was combined with a quick T-RFLP analysis of the *mcrA* gene as discussed earlier. The metagenome data were evaluated by both read-based and genome-centric bioinformatics tools to obtain taxonomic affiliation of the most abundant taxa and evaluate their changes in relative abundance as a function of substrate change. The two approaches provided similar results regarding the

major trends of methanogenic taxa, and differences were mainly attributed to the PCR bias-free nature of the metagenome dataset and the imperfect taxonomic identification by T-RFLP (Ács *et al.* 2019). Metatranscriptomics has the advantage of providing information on the metabolically active genes. The first metatranscriptome analysis of a hydrogenotrophic reactor microbiome showed that H_2 injection induced an upregulation of the hydrogenotrophic pathway by increasing the activity of *Methanothermobacter wolfei* UC0008 in a single-stage reactor (Fontana *et al.* 2018). Upregulation of CO_2 fixation pathways producing acetate and butyrate was observed in a two-stage configuration by the most abundant species *Anaerobaculum hydrogeniformans* UC0046 and *Defluviitoga tunisiensis* UC0050. An interesting finding was that the well-known SAOB *Tepidanaerobacter acetatoxydans* was involved in acetate production and uptake, instead of acetate oxidation (Fontana *et al.* 2018).

The metaproteome of microbial communities in hydrogenotrophic reactor microbiomes can be investigated by protein extraction and fractionation followed by chromatographic separation and tandem mass spectrometric analysis (Heyer *et al.* 2015). To exploit the potential of the metaproteomics approach, a more comprehensive hydrogenotrophic microbiome-specific database is needed in order to improve the assignment of the majority of proteins to functions and to reduce the proportion of unknown proteins; however, the biochemical characterization of the enzymes is also essential to confirm their functions. To understand the function and role of these proteins, currently supportive metagenome information as scaffold is suggested (Heyer *et al.* 2017), as limitations of the metaproteomics approach were demonstrated in an earlier study on biogas plants in which several proteins could not be identified (Hanreich *et al.* 2012). Integrated metagenome and metaproteome analyses of a biogas plant showed that public databases yielded insufficient identification rates, compared to a corresponding metagenome

database from the same sample (Ortseifen *et al.* 2016). The application of metaproteomics for biogas plant samples has been reviewed, including an overview of the workflow and potential pitfalls (Heyer *et al.* 2015) especially regarding challenges in metaproteomics data analysis (Muth *et al.* 2013).

A general conclusion from these studies is that mesophilic *ex situ* biogas upgrading or biomethanation of pure H_2 and CO_2 processes are mainly dominated by the genera *Methanobacterium*, while *Methanothermobacter* was the most predominant at higher temperature range. In case of *in situ* biogas upgrading, *Methanoculleus* was also found as an important genus. The bacterial community was always more diverse, and various taxa were suspected to be responsible for the homoacetogenic activity. The genus *Methanothrix*, containing strictly acetotrophic methanogens, was also frequently found as minor member of the hydrogen-utilizing community, probably involved in the conversion of acetate produced by homoacetogenic bacteria (Ács *et al.* 2019; Logroño *et al.* 2020, 2021).

These meta- or multi-omics studies also unveiled that most of the microorganisms are still unexplored and only limited functional information could be derived due to missing reference genome information (Treu *et al.* 2016). Next-generation sequencing technologies are still advancing rapidly and substantial cost reduction per read can be expected in future, which will further accelerate the application of omics approaches in the field of bioreactor microbiology research. Metabolomics is not discussed in this review in detail because in P2X systems metabolite analyses are restricted only to the major useful products and complex studies are missing to date. The analyses of the gas composition and VFA concentration measurements are standard methods and can be considered a basic version of metabolomics providing useful information about the competition of homoacetogenesis and methanogenesis in P2G systems. In

addition, future P2X technologies in food sector might include the microbial production of food additives, e.g., terpenoids (colors and antioxidants), probiotic effector compounds etc., which will require advanced analytics of the metabolites. Methods based on substrate-mediated isotope labelling of nucleic acids or proteins in combination with modern molecular or single cell techniques have been successfully used in microbial ecology (Pumphrey *et al.* 2011; Jehmlich *et al.* 2016; Mosbæk *et al.* 2016; Wang *et al.* 2019; Zheng *et al.* 2019). Protein-SIP in combination with metagenome analysis was performed on the microbial community of an *in situ* biomethanation process in CSTRs inoculated with sludge from a biogas reactor treating manure and dairy waste and fed with glycerol. The biomass was labelled by adding $NaH^{13}CO_3$ to the liquid for metaproteome analysis. The ^{13}C-labelled peptides were mainly related to enzymes involved in genetic information processing, carbohydrate metabolism and transport, but also key enzymes of the Wood-Ljungdahl pathway were detected (de Jonge *et al.* 2022). However, these labelling methods have less relevance in P2X systems, because the whole microbiota relies only on few substrates, e.g. H_2 and CO_2, and longer incubation leads to cross-feeding. Future studies applying multi-omics approaches should go beyond snapshot analyses and need to support complex experiments carefully designed to answer specific ecological questions (Prosser 2015).

The need for cultivation

Linking physiological function to molecular datasets by comparing sequences to closely related cultured relatives has many limitations. Even closely related microorganisms might have completely different functions, and short-read 16S rRNA gene sequences yield genus-level classification at best, which further reduces the predictability of the metabolic function. Moreover, a substantial number of the sequences are related to unknown species. The gap

between the number of cultivated, well described microorganisms and the putative microbial species described only by DNA sequences is widening at an increasing rate. As a consequence, there is a similar gap between the characterized and hypothetical proteins identified only by metagenomics (Hugenholtz and Tyson 2008) and overall a gap between current microbial cultivation and multi-omics approaches (Gutleben *et al.* 2018). The novel cultures would allow detailed biochemical characterization of novel enzymes as well as exploring as-yet unknown physiological traits under varying cultivation conditions. These observations highlight the need for obtaining more key players of hydrogen conversion processes in pure cultures or defined co-cultures by applying novel and more sophisticated cultivation methods. Genome information retrieved from metagenome data (so-called metagenome-assembled genomes – MAGs) can also help in the development of isolation and cultivation strategies (Pope *et al.* 2011). A similar strategy could be applied for the targeted isolation of abundant but so far not cultivated microorganisms of the complex microbiome of hydrogen-fed reactors producing methane and other valuable chemicals or protein-rich biomass. Such efforts are not always needed, and even simpler enrichment and isolation strategies can result in the isolation of novel species. In a specific example, enrichment of HOB was followed by amplicon sequencing of the 16S rRNA gene. The enrichment culture steps always resulted in a diverse mixture of HOB and heterotrophic bacteria. Isolation was performed by standard plating and dilution to extinction approach and revealed several new hydrogen-oxidizing strains belonging to the genera *Paracoccus*, *Achromobacter*, and *Hydrogenophaga* (Ehsani *et al.* 2019). One of the isolated strains was later described as a new species, namely *Achromobacter veterisilvae* (Dumolin *et al.* 2020).

Although there are several MOB and HOB in culture collections, novel strains might have higher protein content, better amino acid composition or could be easier genetically modified. Isolation of novel bacteria for the production and modification of chemicals is also welcomed. These novel isolates could be useful not only for further industrial applications, but also for better understanding the role and function of microbial metabolic traits in natural and engineered systems. Single-culture biotechnology or defined co-cultures can be advantageous for specific applications, but using microbiomes could maintain functionality when complex and fluctuating quality substrate is used or bigger flexibility is required due to their better adaptive capacity to changes.

PERSPECTIVES AND OUTLOOK

The near future perspectives are somehow clear in the electricity sector with the trend of increasing share of renewable energies, especially wind and solar power in the years to come (IEA 2020; Harnisch and Morejón 2021). However, the race for the best and most affordable energy storage is still on, and the P2G approach is a promising contender with already proven technologies for grid stabilization and storage at industrial scale (Schiebahn *et al.* 2015; Götz *et al.* 2016). Novel reactor types improving the gas transfer and optimization of the reactor microbiome are foreseen in the future in case of biomethanation of hydrogen. We do not expect a broad application of genetic engineering in P2G, as it has not been applied in traditional AD technologies (biogas or wastewater treatment sector), but improved microbial resource management with upgraded molecular tools and enriched or synthetic co-cultures can be predicted. However, genetic engineering of microorganisms used in axenic cultures could help improving robustness, efficiency or selectivity of the process. Axenic cultures of

Methanobacterium thermoautotrophicus, for example, are already used for *ex situ* biomethanation. A better understanding of the syntrophic and competing interactions among microorganisms in reactors may provide means of better management and hence process performance and stability (De Vrieze *et al.* 2018; De Vrieze *et al.* 2020a; Verstraete *et al.* 2022). The role of seeding inocula should be further studied and emphasized, and even a trade of optimized system-adapted inocula for start-up of new biomethanation systems seems interesting and may even result in a business model. The advantages and limits of flexible operation modes should be further investigated and the ecological strategies behind the adaptation of the microbiota should be understood to maintain good technical performance, flexible functionality, and resilience of the whole reactor microbiome (Logroño *et al.* 2021).

In the case of pure culture or few-membered mixed culture strategies, especially in Power-to-Chemicals or Power-to-Protein approaches, we expect the utilization of genetically modified organisms (GMO). Autotrophic feed with gases could be easily kept in axenic cultures since the risk of contamination is low, and the inactivation of the GMOs is easy in order to avoid their unintentional release to the environment.

Another paradigm shift can be expected in the food sector by the widespread implementation of microbial technologies for protein production from hydrogen or methane. Direct utilization of hydrogen or a two-step approach combining electrochemical or biological conversion of hydrogen to another, easy to utilize substrate, *e.g.*, methane, methanol, acetic acid, formic acid, which can be fed into a second MP-producing reactor, are alternative options (Mishra *et al.* 2020). Although the technology is not novel, relatively few hydrogenotrophic autotrophs have been isolated and analyzed in detail. Thus, we see a grand potential for establishing new production strains, as besides genomic and metagenomic information, the availability of pure

cultures for such an approach is urgently needed. Therefore, additional efforts are required to explore new habitats for the isolation and description of new strains. A better portfolio of available microorganisms (Fig. 2 and 4) is required for improving cell factories for custom-designed protein production. Such designed food proteins, combined with the newest technologies of artificial meat production (Bhat *et al*. 2015; Bonny *et al.* 2015), can replace current animal farming practices with huge land, water and antibiotics requirements. A renewed interest in space exploration may also enhance research toward MP production as potential means of food production during long-term space missions, at space stations or for colonizing other planets (Clauwaert *et al.* 2017). However, as a first step, MP could be used as animal feed providing comparably priced and even better-quality alternatives of current fodders regarding protein content and amino acids composition. Global climate change strongly influences the fodder prices on the world market today, which consequently influences the overall price of meat production. Beside methane and protein production, combined microbial electrochemical approaches to produce simple organic molecules, such as formate and volatile fatty acids, also in combination with subsequent biosynthesis to form more complex molecules such as polyhydroxybutyrate, terpene or polymer bricks, are on their way. Here, one challenge is the combination of electrochemical and biological synthesis in one system as optimal process conditions mostly differ (*e.g.*, salt concentration and pH). Further, the different rates (*i.e*., space time yields) of microbial and electrochemical processes may allow exploitation of "peak" currents.

We are witnessing the emergence and sometimes re-invention of biotechnological processes addressing major societal challenges. Further improvements of microbial resource management and exploration of novel natural bioresources are needed to go beyond laboratory or pilot-scale

demonstrations with extended techno-economic and environmental assessments to achieve sector disruptive technologies. This includes, *e.g.*, the replacement of current livestock industry, new achievements in energy storage and CO_2-neutral chemical building blocks. P2X biotechnologies are able to address these challenges in a sustainable way as they i) follow principles of green chemistry, e.g., increase the atom economy (i.e. most of the atoms of the reactant are incorporated in the desired products) by using CO_2 in biogas, operate at nearly ambient temperature and pressure, use aqueous solvents, ii) recycle CO_2 and iii) are able to operate on 100% renewable power and substrates. Therefore, we expect P2X to become one major biotechnological route of the near future.

State-of-the-art biotechnology profits from the integration of biology, bioinformatics, engineering and renewable energy. Biology provides the ground for exploration and development of bioprocesses, bioinformatics helps elucidate the functional potential of not yet cultured microorganisms, engineering allows process optimization and techno-economic assessment, and the integration of renewable energy with those processes enables the transition to the sustainable production of goods for different industries.

The way from samples to enrichment cultures, DNA sequences and metabolic models is very laborious. Currently, the task of reconstructing and annotating genomes and MAGs involves several steps of manual work until a valid genome-scale metabolic model is obtained to explore the functional potential of the respective microorganism or a microbial consortium. Developing more automatized pipelines in systems biology would enable the discovery of new products, new pathways and new production hosts. Exponential gains might be possible through an integrative approach of different tools and disciplines, thus, allowing humankind to benefit from the biotechnological potential of microbes. With the current development of robotics and artificial

intelligence it could be possible to increase our ability to process samples from many more underexplored environments at unprecedented speed.

Omics tools offer unprecedented resolution to decipher the functioning of complex communities, thus they could help develop guided principles for designing multi-species cultures with desired metabolic functionalities. This could have great impact on the chemical industry since various microbes could be coupled to exploit their native biosynthetic pathways to produce biochemicals. Taking the example of biomethanation of hydrogen, it has either been done with microbial communities or pure cultures. Considering that various methanogens have different levels of affinity to hydrogen concentrations it is conceivable to use abundance data of methanogens from microbial communities to design methanogenic multi-species cultures.

Pushing the boundaries to explore new potential applications beyond the obvious physiological products would enable us to find disruptive solutions. For instance, a recent study found that methanogens were able to produce lipids or to excrete proteinogenic amino acids (Taubner *et al.* 2019). This has even sparked entrepreneurial activities. For instance, new companies like Arkeon GmbH are exploiting this biotechnological process and paving the way for the production of all essential amino acids as ingredients for the food industry from H_2 and CO_2 using a proprietary methanogenic strain. This is a revolutionary technology that could help make the food system more sustainable.

Although these are particular examples, it is clear that not only microbial communities but also pure cultures should be explored in more detail beyond traditional microbiological descriptions in order to move beyond the status-quo. Curiosity-driven research coupled with high throughput analytical methods and automatization could pave the way for accelerated discovery at

unprecedented speed to provide sustainable solutions in the food, chemicals and energetic sectors on a changing planet.

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Table 1. Community members of different stages of anaerobic digestion

Stage	Substrates	Products	Microorganisms (various taxonomic levels)	Reference
Hydrolysis	Carbohydrates, lipids, proteins	Sugars, amino acids, long-chain fatty acids	*Clostridium*, *Ruminococcus*, *Caldicellulosiruptor*, *Acetivibrio*, *Butyrivibrio*, *Halocella*, *Fibrobacter*, *Bacteroides*, *Spirochaeta*	(Azman *et al.* 2015) (Bayané and Guiot 2011) (Lynd *et al.* 2002)
Acidogenesis	Sugars, amino acids	VFA, alcohols lactate, hydrogen and carbon dioxide	*Petrimonas* *Paludibacter* *Clostridium*, *Lactobacillus*, *Streptococcus, Ruminococcus* *Bacteroides* *Propionibacterium*	(Grabowski et al., 2005 (Ueki et al., 2006) (Bayané and Guiot 2011) Alvarado et al., 2014
Homoacetogenesis	H_2, CO_2	Acetate	Homoacetogens are affiliated to Clostridiales, Selenomonadales and Thermoanaerobacterales	(Schiel-Bengelsdorf and Dürre 2012; Bengelsdorf *et al.* 2018)
Syntrophic acetate oxidation	Acetate	H_2,CO_2, formate	*Pseudothermotoga* *Thermacetogenium* *Clostridium ultunense* *Syntrophaceticus* *Tepidanaerobacter*	(Müller *et al.* 2016; Westerholm, Moestedt and Schnürer 2016)
Syntrophic propionate oxidation	Propionate	Acetate, CO_2, H_2, formate	*Smithella* *Pelotomaculum* *Syntrophobacter* Ca. Propionivorax syntrophicum	(Worm *et al.* 2014) (Hao *et al.* 2020)
Syntrophic butyrate oxidation	Butyrate	Acetate, CO_2, H_2, formate	*Syntrophomonas* *Syntrophus* *Syntrophothermus* Ca. Phosphitivorax anaerolimi Ca. Phosphitivorax butyraticus	(Schink and Stams 2006; Worm *et al.* 2014; Sieber *et al.* 2018) (Hao *et al.* 2020)
Hydrogenotrophic methanogenesis	H_2 and CO_2 or formate	Methane	Methanobacteriales Methanocellales Methanococcales Methanomicrobiales Methanosarcinales	(Berghuis *et al.* 2019)
Acetotrophic methanogenesis	Acetate	Methane, CO_2	Methanosarcinales	(Berghuis *et al.* 2019)
Electron mediated methanogenesis	CO_2, electrons and protons (EET)	Methane	Methanobacteriales *Methanococcus maripaludis* Methanosarcinales	(Zheng *et al.* 2020) (Lohner *et al.* 2014) Rotaru *et al.* 2014; Yee *et al.* 2019; Yee and Rotaru 2020)
Methylotrophic methanogenesis	Methylated compounds (methanol, methylamines, and methylsulfides)	Methane	Methanobacteriales Methanomassiliicoccales Methanosarcinales	(Berghuis *et al.* 2019)

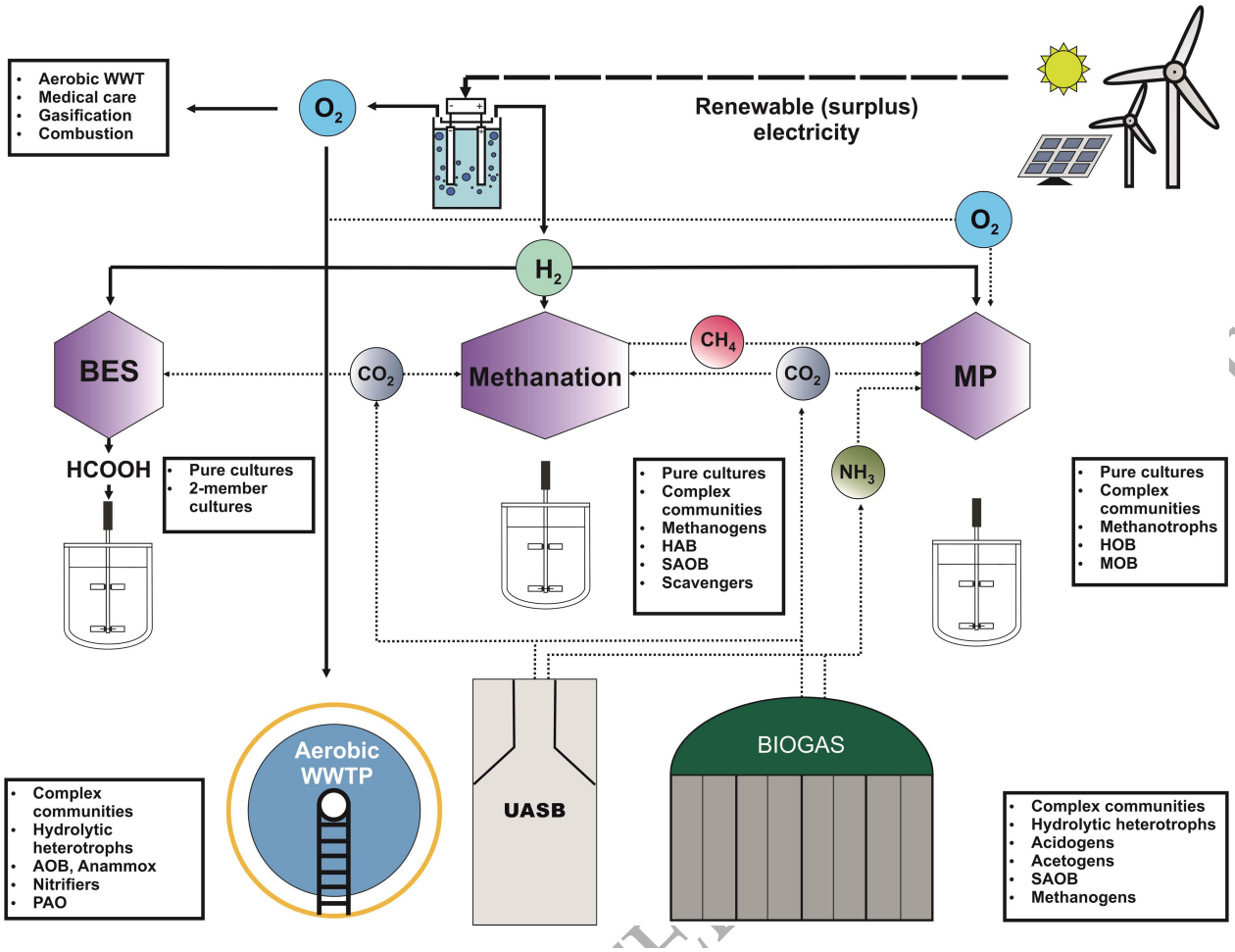
VFA: volatile fatty acids; EET: extracellular electron transfer

Table 2 Overview of selected studies in which either pure or enriched cultures were used for microbial protein production using the hydrogen route. HOB = hydrogen-oxidizing bacteria, DW = dry weight. NA = not available.

HOB	Culture type	Protein content (% DW)	Reference
Cupriavidus necator[1]	Pure	95	Repaske and Mayer (1976)
Sulfuricurvum spp.	Enriched	71	Matassa *et al.* (2016b)
Paracoccus denitrificans Y5	Pure	67-74	Dou *et al.* (2019)
Paracoccus versutus D6	Pure	67-74	Dou *et al.* (2019)
Azonexus spp.	Enriched	62-72	Hu *et al.* (2020)
unclassified *Comamonadaceae*	Enriched	62-72	Hu *et al.* (2020)
Achromobacter veterisilvae	Pure	NA	Dumolin *et al*. (2020)

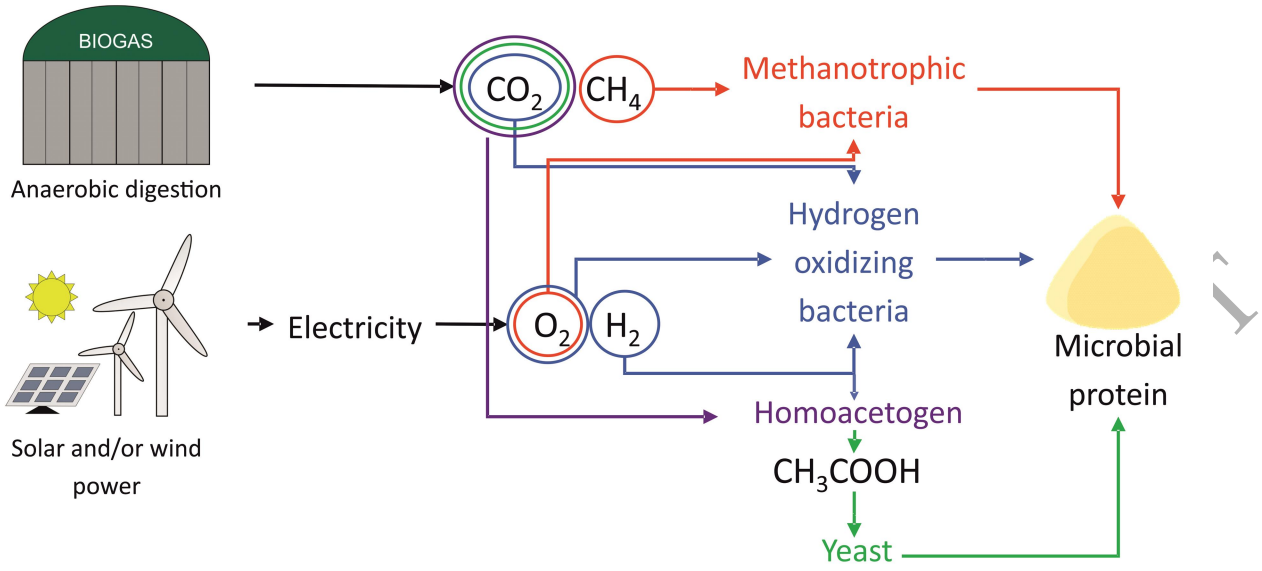
[1]This species underwent multiple changes in taxonomy and was formerly named as *Alcaligenes eutrophus* (Khosravi-Darani *et al*. 2013, Matassa *et al.* 2015b).

Figure 1



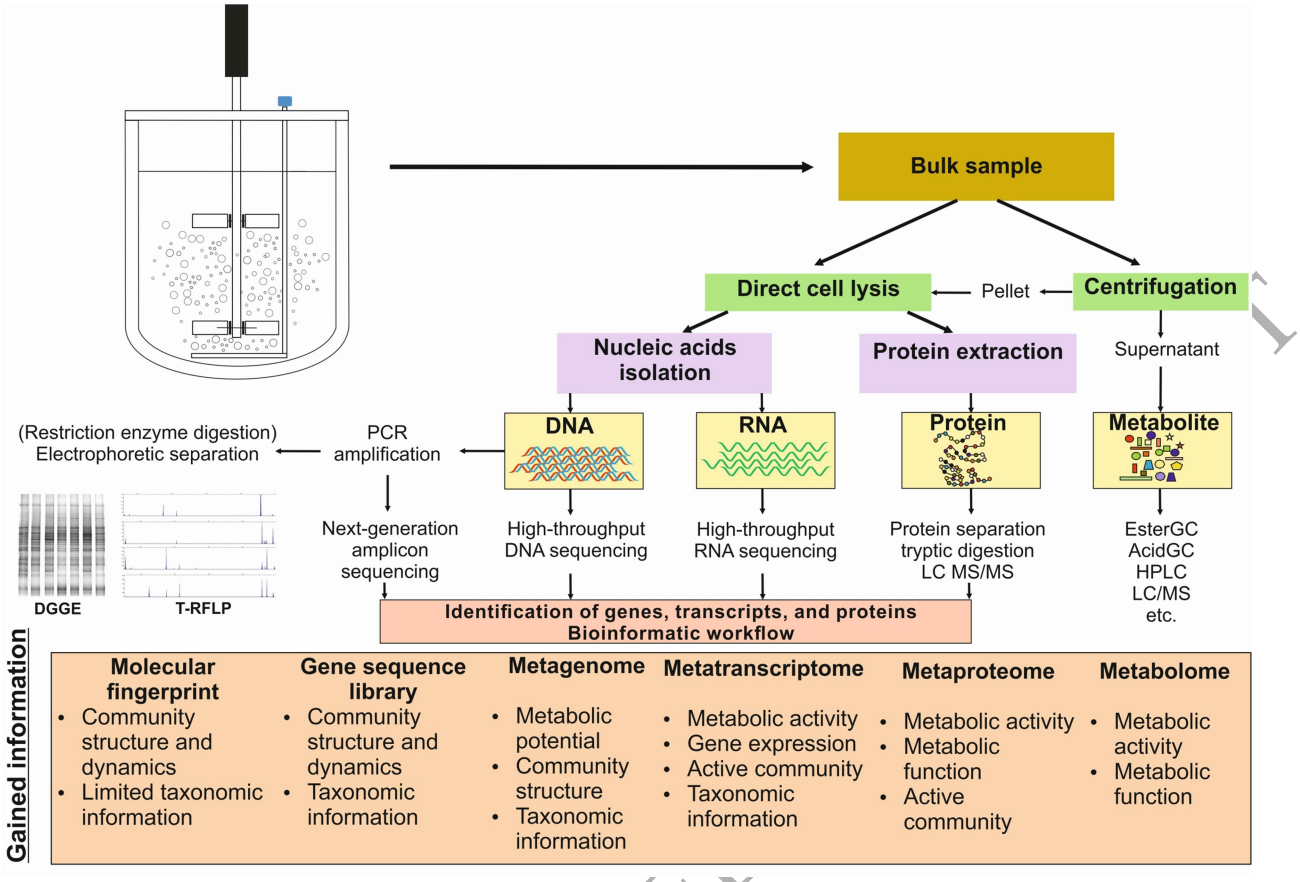
P2X systems to use renewable green hydrogen. WWT: wastewater treatment; WWTP: wastewater treatment plant; UASB: upflow anaerobic sludge blanket; BES: bioelectrochemical systems; HOB: hydrogen-oxidizing bacteria; MOB: methane-oxidizing bacteria; HAB: homoacetogenic bacteria; SAOB: syntrophic acetate-oxidizing bacteria; AOB: ammonia-oxidizing bacteria; PAO: phosphorus-accumulating organism.

Figure 2



Potential ways of coupling anaerobic digestion and the renewable power sector for microbial protein production through different routes.

Figure 3



Various classical molecular and omics approaches to investigate the microbiology of P2X systems, including the level of information that can be obtained.

Figure 4