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Advancement, challenges, and future roadmaps

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From renewable energy to sustainable protein sources: Advancement, challenges, and future roadmaps

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ABSTRACT

The concerns over food security and protein scarcity, driven by population increase and higher standards of living, have pushed scientists toward finding new protein sources. A considerable proportion of resources and agricultural lands are currently dedicated to proteinaceous feed production to raise livestock and poultry for human consumption. The 1st generation of microbial protein (MP) came into the market as land-independent proteinaceous feed for livestock and aquaculture. However, MP may be a less sustainable alternative to conventional feeds, such as soybean meal and fishmeal, because this technology currently requires natural gas and synthetic chemicals. These challenges have directed researchers toward the production of 2nd generation MP by integrating renewable energies, anaerobic digestion, nutrient recovery, biogas cleaning and upgrading, carbon-capture technologies, and fermentation. The fermentation of methane-oxidizing bacteria (MOB) and hydrogen-oxidizing bacteria (HOB), i.e., two protein rich microorganisms, has shown a great potential, on the one hand, to upcycle effluents from anaerobic digestion into protein rich biomass, and on the other hand, to be coupled to renewable energy systems under the concept of Power-to-X.

This work compares various production routes for 2nd generation MP by reviewing the latest studies conducted in this context and introducing the state-of-the-art technologies, hoping that the findings can accelerate and facilitate upscaling of MP production. The results show that 2nd generation MP depends on the expansion of renewable energies. In countries with high penetration of renewable electricity, such as Nordic countries, off-peak surplus electricity can be used within MP-industry by supplying electrolytic H₂, which is the driving factor for both MOB and HOB-based MP production. However, nutrient recovery technologies are the heart of the 2nd generation MP industry as they determine the process costs and quality of the final product. Although huge attempts have been made to date in this context, some bottlenecks such as immature nutrient recovery technologies, less efficient fermenters with insufficient gas-to-liquid transfer, and costly electrolytic hydrogen production and storage have hindered the scale up of MP production. Furthermore, further research into techno-

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economic feasibility and life cycle assessment (LCA) of coupled technologies is still needed to identify key points for improvement and thereby secure a sustainable production system.

1. Introduction

The increase in global temperature, caused by climate change, has become a formidable challenge so that United Nations' IPCC Special report has encouraged the implementation of multidisciplinary actions by 2030 before climate change becomes irreversible [1]. Anaerobic digestion (AD) and in particular biogas industry, regardless of its prominent role for renewable energy production and waste management, is becoming a key player for decreasing global warming impacts and limiting it to 1.5 °C by 2100 [2]. Hence, the number of biogas plants have been rapidly increasing worldwide. Currently, thousands of biogas plants (almost 132,000 plants at the time of writing this paper) are operating across the world [3] producing biomethane and biogas slurry (digestate) as co-products. While the produced biogas can be either injected into the gas pipelines (subject to biogas upgrading) or combusted to generate heat and electricity [4,5], the use of digestate as organic fertilizer is challenging due to its bulky nature [6,7].

Although the remaining digestate after AD has been known as a macro- and micro-nutrient rich soil conditioner, some challenges such as storage during off-season, transportation cost, presence of soil contaminants, and high mechanization requirements for field application have decreased its popularity and hindered its wide application by farmers especially in non-industrialized countries [2,8]. On the other hand, ammonium comprises more than 60% of the nitrogen in digestate, which is prone to ammonia volatilization right after field application. Moreover, a considerable fraction of this ammonium nitrogen is also prone to leakage or run-off to water bodies [9]. Furthermore, nitrogen use efficiency by plants is reportedly below 50% meaning the remaining half contributes to eutrophication, emissions of the potent greenhouse gas N₂O, and degradation to nitrate (a contributor to eutrophication) or nitrite (toxic for most life forms) [10,11]. Such problems have motivated scientists to focus on nutrient recovery from digestate for valorization into value-added products or being simply used as less-bulky fertilizers [9,12,13].

Microbial protein (MP), as a cutting-edge technology, can be considered as the nexus of clean energy and food security. On the one hand, it can assimilate nitrogen from digestate in the form of protein and on the other hand produce a protein-rich biomass competitive with conventional protein sources such as soybean meal and fishmeal [14–16]. In the 1980s, scientists made efforts to produce high-quality protein-rich microorganisms which can be used as food/feed ingredient, named MP [17]. Among different types of MP (i.e., algae, yeast, bacteria, and fungi), bacterial-based MP such as methane-oxidizing bacteria (MOB) [18–21] and hydrogen-oxidizing bacteria (HOB) [22–26] have gained more interest because of their high protein content, digestibility, and amino acid profile comparable with conventional proteins like soybean meal [14,15,27]. The low growth rate and low protein content of fungi and yeast-based protein sources, non-digestible cellulosic cell wall as well as concentrate heavy metals of algae, and high nucleic acid content and low density of bacteria gave them low popularity as future protein sources compared to MP [20,28]. For instance, MOB is a MP that uses methane as carbon and energy source while assimilating nitrogen in the form of protein [20,21]. The commercialized MOB-based MPs such as UniProtein and FeedKind [29,30] can be considered as 1st generation types since they use chemical nitrogen and natural gas as their nitrogen and energy sources, respectively. So called 2nd generation MPs substitute synthetic nutrients and natural gas in this process with their counterparts from waste effluents such as nitrogen from digestate and methane from biogas thereby reducing cost and contributing to the sustainability of MP production [23,31].

The valorization of nutrients from organic waste into high quality

MP can be performed via different routes by combining well-established technologies namely AD, biogas cleaning and upgrading, nutrient recovery, and fermentation (Fig. 1). To establish the most sustainable platform for organic waste valorization into MP, these possible routes and the associated technologies should be well understood, and their sustainability should be holistically assessed. Accordingly, this paper aims to review the established and state-of-the art technologies that can match the concept of integrating renewable energy and MP production to introduce the most sustainable routes for 2nd generation MP production, facilitate scaling up the technology, and indicate future roadmaps. Our literature review shows that previous studies conducted in this context attempted to demonstrate the proof-of-concept at lab scale and kept their focus on a narrow aspect of the technology (Table 1). To the best of our knowledge, this paper is the first attempt to comprehensively scrutinize the above-mentioned technologies within the concept of integrated renewable energy production and MP production. The results will show the gaps in the field and the future roadmaps for both researchers and policy makers and will contribute to food security and sustainable development goals. The findings of the present study are in agreement with the Sustainable Development Goals (SDG's) for the year 2030 which have been agreed by all members of the United Nations [32]. In particular, Goal 2 is to stop starvation, Goal 15 is to fight climate change, and Goal 17 is to protect life in water and on land. Hence, in order to meet the SDG's objectives, it will be ideal to find sustainable and biobased protein sources.

2. Production routes for 2nd generation MP

Methane-oxidizing bacteria (MOB, also known as methanotrophs) and hydrogen-oxidizing bacteria (HOB, also known as hydrogenotrophs) have been reported as the most suitable microorganisms for upcycling nitrogen into MP due to their high protein content [15,23,25,30,34,45]. HOB are grown on H₂, O₂, and CO₂ with additional substances as supplementary nutrients (Fig. 2-A). Agro-urban wastes undergo AD process to produce biogas and stabilize the organic wastes. The generated biogas after AD contains 40–70 v/v% CH₄, 30–60 v/v% CO₂, 0–1 v/v% H₂, 0–3 v/v% H₂S, and 0–5 v/v% other gases [46,47]. The biogas is subject to purification and upgrading by which methane is separated and injected to natural gas grid, being as a home cooking or transportation fuel, or combusted in combined heat and power (CHP) plants to generate heat and electricity [4,5]. The remaining carbon dioxide after biogas upgrading (depending on the upgrading technology employed for this purpose) can be injected to fermenters to be used as a carbon source for HOB. Simultaneously, the liquid fraction of digestate should undergo a nutrient recovery process (such as stripping [48], electrochemical extraction [19,36], microbial electrochemical extraction [15], or struvite production [6,49]). The recovered nitrogen can then be used as a nitrogen source for MP fermentation where it is assimilated by bacteria and transformed into protein. Other extracted nutrients such as phosphorous can be also used as supplementary cultivation media. Digestate should be treated and purified before being added to the fermentation tanks creating few challenges. Solid residues should be discarded and then, the liquid fraction should be diluted in order to adjust the N levels [20]. However, these process steps can lead to losses of essential micro-elements and trace elements. For instance, lack of copper in the used digestate can lead to decreased MOB growth rate [15]. Trace element deficiency may occur due to the pre-processing of the digestate through centrifugation, filtration or dilution to achieve the desired N levels. To avoid nutrients shortage, microbial adaptation to higher N levels can be gradually applied as a means to decrease the dilution rates [50]. Hence, micro-nutrients availability should be closely monitored

before digestate is used for MOB/HOB cultivation. The required H_2 and O_2 can be supplied via well-established electrolyzers using renewable electricity sources [24,29].

MOB use methane as both a carbon and energy source while assimilating nitrogen to protein [20,21]. After upgrading (CO_2 removal), the biogas contains biomethane with high purity, which can be used as carbon source for MOB. The generated biomethane is then fed into fermenters to supply MOB with energy and carbon (Fig. 2-B). As in the HOB production route, the recovered nitrogen from the liquid fraction of the digestate can be used as a nitrogen source for protein production. It is worth mentioning that the amount of methane needed in this process depends on the available ammonium nitrogen for MP production. It has been reportedly shown that a methane-to-ammonium nitrogen ratio of 7–14 can ensure a high-quality MP with up to 67% pure protein [19,20]. Therefore, how much biogas (or biomethane) from a biogas plant should be directed to MP production depends on the feedstocks which are processed in the biogas plants. For instance, regarding a biogas plant mono-digesting biopulp (i.e., source sorted organic fraction of municipal solid waste treated with pulper technology [40]). With a TS of 18.3%, 176.48 kg biogas per tonne of biopulp (with 60% methane) can be produced. One tonne of this biopulp after anaerobic digestion contains 2.71 kg ammonium nitrogen. Accordingly, 28.45% of biogas is needed to upcycle the available ammonium nitrogen into MP. If this biogas is biologically upgraded into biomethane, 103.74 kg biomethane per tonne of biopulp will be available. Under such circumstances, 28.45% of the initial biogas or 17.78% of upgraded biogas would be enough to valorize 2.71 kg ammonium nitrogen to MP [33]. As can be seen, for a given amount of biopulp, only a small fraction of its biogas/methane potential is needed to upcycle its nitrogen content into MP (details can be seen in supplementary file SI-1). However, for feedstocks with high nitrogen content but low biodegradability the situation can be different. Considering anaerobic digestion of sludge from wastewater treatment

plants, 41.14% extra biogas than plant's total production is needed to fully valorize its nitrogen stream into MP. If this biogas plant is coupled with biological biogas upgrading system, 84.68% of its total methane production should be used within MP facility. The use of biogas/biomethane within MP facility instead of their conventional use such combustion in biogas engines to produce heat and electricity or their use as transportation fuel to a large extent can be regulated by the market price of biogas for either application. Regional policies on energy schemes or subsidies on biogas for specific applications can affect the final application of biogas.

Another possible route which is more suitable for countries with surplus renewable electricity production, such as Denmark and Germany, is shown in Fig. 2-C. Under such a scenario, biological biogas upgrading, which is part of the Power-to-Gas technology, is regarded as the cutting-edge upgrading technology that uses surplus electricity from renewable resources for electrolytic hydrogen production [51]. Under such circumstances, the biogas is treated with biological biogas upgrading to convert CO_2 content of biogas into natural gas grade biomethane through chemoautotrophic reactions [46,52,53]. The generated biomethane is then fed into fermenters to supply MOB with energy and carbon.

Biogas upgrading, depending on the technology type, is subject to the consumption of chemicals, use of heat and energy, operating and maintenance costs, etc. The concept of consecutive MOB-HOB fermentation can, to some extent, compensate investment and operating costs associated with biogas upgrading by providing more income and additionally improve the environmental profile of 2nd generation MP by decreasing energy and materials requirement. In this concept the biogas used for MP production will not be treated by upgrading technologies. Under such circumstances the produced biogas, after moisture and H_2S removal, can be fed into MOB reactors to feed them with methane. The off-gases from MOB fermenters are mostly composed of CO_2 with small

Fig. 1. A generalized platform for microbial protein production from agro-urban waste. T1 to T4 represents the challenges for selecting the best technology or management practices for sustainable microbial protein production: T1 = anaerobic digestion; T2 = upgrading technology; T3 = nutrient recovery; T4 = fermentation and post-processing; T5 = other supplementary technologies.

Table 1

A summary of previous studies conducted to scrutinize technical/economic/environmental aspects of MP production.

Year	Location	Waste fraction	Functional unit	Scale	Sustainability assessment			MP	MP yield	Mode		Bacteria growth substrate feedstock						Refs.
					Environment	Economic	Technical			Batch	CSTR	H ₂	O ₂	CO ₂	CH ₄	NH ₄ /NH ₃	Further nutrients	
2021	Denmark	Wastewater	27 mill m ³ wastewater	CSR	+	–	–	MOB	–	+	–	–	Air	–	AD/NG	Reject water	IOC	[33]
2019	Denmark	Household waste	–	LS	–	–	+	MOB	0.4–0.7 ^b	+	+	–	CAS	–	AD/NG	Syn/Dig	IOC	[34]
2019	Na	Chicken manure	–	LS	–	–	+	HOB	6.5 ^a	+	–	Na	Na	Syn	–	Syn	IOC	[35]
2016	Belgium	Food (potatoes)	–	LS	–	–	+	HOB	Na	+	+	Elect	Elect	Syn	–	Syn	IOC	[26]
2017	Na	Urine	–	LS	–	–	+	HOB	Na	+	–	Elect	Elect	Syn	–	Urine	IOC	[36]
2017	–	Na	–	VS	–	–	+	MOB	Na	–	+	–	–	–	–	–	–	[37]
2020	–	Na	–	LS	–	–	+	MOB	0.57 ^b	+	–	–	CAS	–	NG	Syn	IOC	[38]
2016	China	Na	–	LS-PS	–	–	+	MOB	Na	–	+	–	Air	–	NG	Syn	IOC	[39]
2020	China	Wastewater	–	LS	–	–	+	HOB	Na	+	+	Elect	Elect	Syn	–	Syn	IOC	[24]
2021	Copenhagen	OFMSW	1 t biopulp	VS	+	+	–	MOB	9.8 ^a	–	–	Elect _w	Elect _w /CAS	–	AD	Dig	–	[40]
2020	Belgium	Pig manure and pumpkin	–	LS	–	–	+	MOB	Na	+	–	BUE	BUE	AD	AD	Syn	IOC	[41]
2020	Na	Na	–	LS	–	–	+	MOB/HOB	5.2–6.9 ^a	–	+	–	CAS	–	NG	Syn	IOC	[42]
2020	Denmark	Wastewater	–	LS	–	–	+	MOB	Na	+	–	–	CAS	–	NG	Syn	IOC	[43]
2020	Europe	Wastewater	1 kg MP	VS	+	–	–	HOB	Na	Na	Na	Elect	–	Waste	–	Syn	IOC	[29]
2011	Na	–	1 t protein	CSR	+	–	–	MOB	Na	–	+	–	CAS	–	–	Syn	IOC	[44]
2021	Denmark	Sewage sludge	–	LS	–	–	+	MOB	11.5 ^a	+	–	–	CAS	–	AD/NG	Dig	–	[14]
2020	Na	Piggery	–	VS	–	+	–	MOB/HOB	0.76 ^b	–	–	Elect _s	CAS/PSA	AD/CHP	AD	Dig	–	[30]
2017	Denmark	Household waste	–	LS	–	–	+	MOB	0.4–1.8 ^b	+	+	–	CAS	–	AD	Syn/Dig	IOC	[15]
2019	Denmark	OFMSW	–	LS	–	–	+	MOB	6.8–11.4 ^a	+	–	–	CAS	–	AD/NG	Syn/Dig	IOC	[20]
2020	Denmark	Synthetic urine	–	LS	–	–	+	MOB	9.7 ^a	+	–	–	CAS	–	NG	Dig	IOC	[19]
2020	Copenhagen	OFMSW	1 t biopulp	VS	+	–	–	MOB	9.8 ^a	+	–	Elect _w	Elect _w /CAS	–	AD	Dig	–	[7]
2021	Finland/Morocco/Iceland	Wastewater	1 kg MP	CSR	+	–	–	HOB	Na	–	+	Elect	Elect	CAS	–	Syn	IOC	[22]

^a g-MP. g^{–1}-NH₄-N.^b g-MP. g^{–1}-CH₄; LS = Lab-Scale; PS = Pilot-Scale; MP = Microbial protein; MOB = Methane Oxidizing Bacteria; HOB = Hydrogen Oxidizing Bacteria; Elect = Electrolytic; Elect_w = Wind electrolytic; Elect_s = Solar electrolytic; BUE = Biogas Upgrading by Electrochemical; CAS = Cryogenic Air Separation; PSA = Pressure Swing Adsorption; Syn = Synthetic; Dig = Digestate; IOC = Inorganic Component; AD = Anaerobic digestion; CSR = Case Study Report; VS = Virtual scenarios; OFMSW = Organic Fraction of Municipal Solid Waste; Na = Not available.

Fig. 2. Schematic diagram of N, C, and nutrient flows and the associated processes for microbial protein production using HOB and MOB.

fraction of O_2 and high purity CH_4 , hence, suitable to be fed into the HOB fermenters (Fig. 2-D). Although, such a concept has not been scrutinized so far, the final product of such a biorefinery would have a wider spectrum of amino acid profile since it is composed of both HOB and MOB and may have a higher market value.

The choice of technology is site-dependent and varies from one location to another. Apart from the main technologies, involved in the above-described routes (i.e., AD, nutrient recovery, biogas cleaning and upgrading, and fermentation which will be discussed in the subsequent sections), some supplementary technologies should be also taken into

consideration to guarantee a sustainable route for 2nd generation MP. The availability and possibility of integrating such supplementary technologies with 2nd generation MP production would affect the final decision over HOB vs MOB route. HOB require hydrogen molecules as electron donors [23]. From the sustainability point of view, the process will be environmentally friendly when hydrogen is electrolytically supplied from wind or solar electricity, especially when using surplus renewable electricity [16,29]. These parameters should be considered when opting the most sustainable platform for MP production. In regions with many biogas plants or with small land availability for the utilization of nutrients from biogas slurry, digestate disposal will be a serious challenge. Under such circumstances, MP production can be regarded as practical solution for digestate management to avoid nutrient discharge into the water bodies and environment.

3. Microorganisms and protein quality

Microorganisms play the key role in fermentation of MP. As MOB and HOB function differently in upcycling waste effluents into MP, they are discussed separately in the following subsections.

3.1. MOB grown on renewable energy and residual resources

Different cell factories have already been explored for the biotransformation of renewable energy. MOB are the well-known biological sink for CH_4 oxidation, which can grow on biogas using CH_4 as carbon source and assimilate nitrogen in form of proteins with quality to be used as feed supplement for animals [54].

It is considered easier to predict and optimize efficient biocatalysis in pure cultures due to precise knowledge of the culture response to metabolic regulation mechanisms, compared with mixed-culture fermentations [55]. However, pure cultures have shown lower affinity to substrate, having higher inhibition levels to potential inhibitors and also to auxotrophy compared to the growth of mixed methanotrophic consortia [56]. For example, if methanol dehydrogenase (MDH) is inhibited, the fermentation of pure cultures can lead to methanol accumulation [57], ceasing MOB growth. On the contrary, mixed cultures can alleviate the challenges triggered by inhibitor accumulation, since a consortium member can take up the inhibitor(s) produced by another co-worker, stabilizing the MP production process. For example, *Methylobacter* and *Methylophilus*, enriched natural communities show potential for high CH_4 assimilation and protein production due to the creation of syntrophic association [15]. Having created a highly efficient synergy, *Methylobacter* spp. Harbors the responsible genes for CH_4 oxidation to methanol, and *Methylophilus* spp. Encodes methanol-oxidation genes which are absent in the former strain. At the same time *Methylophilus* spp. Is responsible for decreasing methanol concentration and thereby alleviates the product inhibition which would negatively affect growth of *Methylobacter* spp.

Apart from the inhibition aspect associated with metabolic intermediates, another big challenge is the presence of H_2S in raw biogas. The adverse impacts of H_2S on MP production have already been reported [34,58,59] revealing a varying IC_{50} (e.g., ~200–1400 ppm). Apart from critical H_2S concentration, the concentration of methane is also important because CH_4 oxidation is more affected in lower CH_4 levels (i.e., 5%) than higher concentrations (i.e., 20% and 50%) [60]. The acceptable tolerance limits are also important for industrial biotechnological applications because gas feeding should also comply with the safety regulations. More specifically, CH_4 in oxygen-rich environments is explosive with a flammable range between 5 and 17% CH_4 in atmospheric air. Up to now, desulfurization is suggested to be applied on biogas downstream of AD [34]. As an alternative and to avoid a desulfurization step, MOB with ability to oxidize H_2S can be explored for MP production in the future. For example, *Verrucomicrobium* MOB should be examined due to their ability to encode sulfide:quinone oxidoreductase to oxidize H_2S [61]. Similarly, Lee et al. [62] found that

facultative MOB were able to grow on H_2S , regardless of the presence of CH_4 . However, MOB can also be inhibited with high concentrations of H_2S . An IC_{50} value of 1400 ppm H_2S was found to be inhibitory for mixed MOB culture consisting of *Methylomonas* and *Methylophilus* [15].

Regarding micro-nutrients, copper is the most important element affecting MOB growth and is widely discussed in the literature. Cu^{2+} content regulates the most important enzymes for CH_4 oxidation. At high Cu^{2+} -to-MOB ratios, the particulate methane monooxygenase (pMMO) is expressed, which can be produced by all MOB species [56]. As an example, *Methylosinus trichosporium* OB3b was found to operate at a threshold up to 5.64 $\mu\text{mole Cu}^{2+}$ per g-protein for pMMO expression [63]. In contrast, at higher Cu^{2+} the sMMO can drive the CH_4 oxidation process in MOB that express both methane monooxygenases (MMOs). In general, pMMO expression is associated with increased growth rates and higher carbon capture and utilization efficacies [64].

Microbial kinetics vary based on the microbes driving the aerobic CH_4 oxidation. *Gammaproteobacteria* have shown higher growth and CH_4 oxidation rates (0.45 h^{-1} and 30 $\text{mmol g}^{-1} \text{h}^{-1}$) than both *Alphaproteobacteria* (0.09 h^{-1} and 20 $\text{mmol g}^{-1} \text{h}^{-1}$) and *Verrucomicrobia* (0.085 h^{-1} and 10–16 $\text{mmol g}^{-1} \text{h}^{-1}$). Thus, most explored microbes (i.e. *M. capsulatus* and *Methylomonas* spp.) for feed production belong to *Gammaproteobacteria* [64].

On the basis of circular bioeconomy, the characteristics of some types of MOB, which have low tolerance to ammonia [65], can limit their exploitation to use N from ammonia-rich wastewaters (i.e., digestate). There are contradictory conclusions in the literature about the efficient growth of MOB on ammonia [66]. Nevertheless, a recent study on the most well-established strain for MP production showed that *M. capsulatus* can oxidize ammonia to nitrate via a yet unknown pathway [67]. Genomic and transcriptomic studies can further enlighten the conditions under which ammonia assimilation is maximized.

As explained above, the industrial biotechnological applications of MOB are mainly focused on the production of biomass for animal feed formulations [68]. Regarding product quality, a recent study compared the commercial FeedKind® (Calysta, USA) with traditional fishmeal revealing the high potential of *M. capsulatus* to be used as an alternative protein source in aquaculture [69]. Valine and arginine were found at higher concentration in MOB, while lysine and histidine were distinctly lower. The rest essential amino acids did not differ markedly with fishmeal. In addition, the Uniprotein (Unibio®, Denamrk) which consists of *M. capsulatus* enriched culture has shown to have a superior composition than fishmeal based on lysine percentage [70]. Moreover, a *Methylophilus* dominated community showed the potential to deliver an essential amino acid pattern similar to high quality fishmeal, having higher metrics of phenylalanine and tyrosine while lysine methionine and cysteine found at lower levels [17]. In accordance with the above-mentioned, the potential of *M. capsulatus* to form a highly proteinaceous biomass is well-defined.

3.2. HOB grown on renewable energy and residual resources

Another group of chemoautotrophs consists of HOB, using H_2 to assimilate CO_2 . HOB cultivation can fully rely on renewable energy, obtaining H_2 from water electrolysis and CO_2 from the discarded fraction of biogas after conventional carbon-capture upgrading processes.

The most well-examined and efficient HOB is *Cupriavidus necator* and the rationale for that is the formation of a highly proteinaceous biomass (>70%) [69,71,72]. *C. necator* uses the RuBisCO enzyme in the Calvin cycle to catalyze the first major step of fixing CO_2 [25]. It has a high potential to grow on residual resources (e.g., wastewaters, reject water, digestate) as it can utilize ammonia and urea which are the common N-sources in these streams [23]. Nickel and iron are the important trace elements for *C. necator* which oxidises H_2 via [NiFe]-dependent hydrogenases. Focusing on pure cultures, Volova and Barashkov [73] explored the amino acid profile and protein content of the *Alcaligenes eutrophus* Z1, *Ralstonia eutropha* B5786, and *Seliberia*

carboxydohydrogena Z1062. All HOB strains had a protein content higher than 70% along with a complete amino acid profile. Interestingly, the examined strains had an amino acid profile similar to casein which is a traditional livestock feed and thus, it was concluded that these HOB could be considered as a potential protein source.

Regarding mixed cultures, Matassa et al. [26] explored an HOB-enriched culture using an aerobic sludge from a potato processing wastewater treatment plant as inoculum. In a chemostat, a microbial community rich in *Sulfuricurvum* spp. Was formed which contained 71% protein. Compared with other bacterial proteins, fishmeal, and soybean meal, insignificant difference was observed among them in terms of amino acid profile, while only arginine content was in shortage when the biomass was compared with soybean meal. Hu et al. [69] used different inocula, obtained from chemostat, a biocathode sludge, and salty sediment to form a mixed HOB community, which was able to grow also on nitrogen gas instead of ammonium. Researchers found that the community was enriched in *Azonexus* and *Xanthobacter* genera, and *Comamonadaceae* representatives when growing on N_2 . Shifting the N-source from ammonium to N_2 gas led to a slightly decreased protein content (from 72% to 62%). Nevertheless, the amino acid composition remained unchanged revealing the potential of producing feed additives 'out of thin air'.

HOB growth characteristics are markedly affected by the added gas feedstock. $\text{H}_2/\text{O}_2/\text{CO}_2$ should be added on proper ratios to stimulate bacterial growth. Tanaka et al. [74] suggested a ratio of 7:1:1, while Volova et al. [75] found an optimum ratio of 7:2:1. Focusing on metabolism, HOB uses O_2 as a final electron acceptor. However, O_2 can devoid hydrogenases activity above certain thresholds, which are different among strains. For instance, a 4% (v/v) can be inhibitory to *C. necator* [23], while Tanaka et al. [74] found that the CO-tolerant *Ideonella* sp. O-1 was robust even at concentrations higher than 30% (v/v). CO-tolerant HOB could be also exploited to upcycle unprocessed syngas containing CO of 20–25% (v/v) increasing the potential of carbon capture, storage and utilization [76].

3.3. Future possibilities for improving MP quality

Overall, both carbon from biogas and/or hydrogen from excess electricity can be exploited for protein production. To better understand how a high-quality protein is produced from renewable resources, more research is needed into the metabolic pathways responsible for protein storage. Genome-scale metabolic modelling can be explored to reveal cell factories for industrial biotechnology [77]. Torre and colleagues developed a flux balance model for *Methylophilus burkatense* 5G aimed at predicting metabolic parameters and avoiding carbon yield reduction [78]. Similarly, Lieven et al. modelled the metabolic network of *Methylococcus capsulatus* to understand the electron transfer to the pMMO [79]. Researchers revealed that the energy released by NH_4 oxidation to NO_2 driven by pMMO affects the O_2 uptake per mol of CH_4 ratio. However, it is very important to avoid NO_2 accumulation due to the risk of inhibition of MOB growth.

Regarding HOB, metabolic modelling can also guide researchers to design strategies for the over-expression of specific genes [25]. Attempts at gene editing *C. necator* include shifting carbon flow from P (3HB) toward isopropanol [80] and over-expression of groESL genes to avoid isopropanol toxicity [81]. Nevertheless, metabolic modelling or genetic engineering of HOB for increasing protein content and improving the amino acid quality has not yet been fully explored. In addition, metabolic engineering approaches on MOB are also limited due to the narrow knowledge on pathways in which they may be activated under certain conditions. Moreover, creating successful metabolic models and predicting key targets for metabolic engineering of methanotrophs have not been successfully achieved yet [82,83]. Especially for MP production, metabolically engineered strains are not yet examined [83]. Despite genetically modified organisms (GMO) not being widely accepted as food additives, this might change in the future especially considering the

increasing needs for proteinaceous food and feed [84].

In the frame of the circular economy, wastewaters appear as an inexpensive N-rich resource for growing MOB and HOB strains [14,35] with desirable amino acid profile (Fig. 3). More attempts on genome-scale metabolic models can lead to deeper understanding of highly-producing microbes grown on ammonium rich streams (e.g., anaerobic digestates) and biogas/biomethane and renewable hydrogen. Regarding the usage of bacterial meal as food additives, sterility and potential content of unwanted compounds such as toxins or nucleic acids is of great importance. Problems related to poor digestibility, gastrointestinal diseases, skin and allergic reactions and other, even more serious problems should be addressed in the laboratory before reaching production units.

4. Nutrient recovery technologies

As in 2nd generation MP production concept, nitrogen and other nutrients are supplied from waste effluents, nutrient recovery technologies will guarantee a safe recovery of process without transmission of pathogens and other harmful organisms. In the following sub-chapters nutrient recovery technologies are discussed in details.

4.1. Common approaches for nitrogen/phosphorus recovery

Digestates contain substantial concentrations of nitrogen and phosphorus which, if not managed properly, lead to strong anthropogenic impacts on aquatic ecosystems [85]. Moreover, due to the growing awareness of the economic and environmental costs of nutrient removal approaches (e.g., biological nitrification/denitrification), recovery technologies have received more attention in recent years due to their benefits in reducing the high cost of nutrient treatment, preventing excessive nutrient loss, and avoiding the generation of reactive nitrogen species (NH_4^+ , NO_2^- , NO_3^-) [86,87]. For example, the treatment costs of the nitrification/denitrification process are estimated at 3400–4000 €. ton^{-1} N converted into nitrogen gas [88], while this can be reduced to 1000–3000 €. ton^{-1} N if commercialized recovery technologies are employed, obtaining products which can be sold at market to yield even larger savings on wastewater management [89].

Nutrient recovery is an important step for upcycling nitrogen-rich waste effluents into value-added products such as MP. Nitrogen-rich waste effluents can, either directly or after pretreatment, be used as cultivation media for microorganisms, such as proteinaceous bacteria (e.g., HOB and MOB), eukaryotic microalgae, and photosynthetic bacteria (e.g., cyanobacteria and), leading to simultaneous nutrient recovery and

production of value-added microbial products (e.g., MP, biofuels and bio-fertilizers) [90,91]. Previous studies demonstrated that macro- and micronutrients as well as trace elements within the digestate make it a nutrient-rich medium for direct cultivation of MP after being centrifuged, filtered, and pasteurized [20,34]. Moreover, the cultivation of microorganisms directly on wastes, especially for food or feed ingredients, would raise safety concerns, and would require new legislations with regard to safety for humans or livestock. Therefore, nutrient recovery for cultivation of MP, should address safety barriers in respect to pathogens, organic micropollutants and heavy metals [50]. Approaches for nitrogen recovery can be divided into physical, chemical, and biological processes (See supplementary file SI-2). Physical processes developed for nitrogen recovery include stripping [92], membrane distillation/separation [93], centrifugation/filtration [94], and physical adsorption/ion exchange [95–97]. Chemical processes for nitrogen recovery are mainly precipitation or electrochemical methods (e.g., electrochemical stripping and electrodialysis) [48,98]. Chemical precipitation is based on the crystallization of struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) as a final product from free ammonium ions [99]. Biological nitrogen recovery could be realized via bioelectrochemical systems (BES) or direct microbial assimilation [90]. BESs have a wide range of applications for waste nitrogen upcycling, which can also be combined with other methods (e.g., membrane separation or stripping) [100].

The most widely used technologies for phosphorus recovery are chemical precipitation or adsorption. Free phosphate can be precipitated into struvite together with NH_4^+ and Mg^{2+} , or form hydroxyapatite ($\text{Ca}_5(\text{OH})(\text{PO}_4)_3$) with Ca^{2+} [101]. The phosphate adsorption can be based on electrostatic attraction, ion exchange, or surface precipitation [102]. A thorough understanding of adsorbent properties and adsorption/desorption behaviour is the key prerequisite to improve phosphate adsorption performance [101]. It should also be mentioned that electrochemical systems have been recently reported as promising methods for phosphorus recovery through mediating precipitation (high local pH at cathode) or driving phosphate separation across the membrane (electrodialysis), which significantly saves operation costs associated with dosing chemicals (in precipitation) or energy consumption (in membrane separation) [103–105]. For this reason, bioelectrochemical systems, rather than abiotic electrochemical systems, have been evaluated as a potential approach to further reduce the energy demand (4 times lower) for phosphorus recovery [106].

Fig. 3. Essential amino acid profile of HOB as reported in Ref. [35] and MOB as reported in Ref. [15] grown on renewable or residual resources compared to fishmeal as reported in Ref. [26].

4.2. AD digestate as a nutrient-rich source

The first step of nutrient recovery from digestate is onsite solid-liquid separation (e.g., screw press, centrifuge, and screening drum press) [107,108]. The solid fraction (with approximately 20–25% TS) contains concentrated levels of dry matter (40–80%), organic nitrogen (20–25% of TN), and phosphorus (40–90% of TP), while the liquid fraction (with approximately 3–7% TS) is rich in ammonia nitrogen [109]. The liquid fraction can be an input stream to an MP production facility. Most of the common nitrogen recovery approaches mentioned above have already been applied to recover nutrients from the liquid fraction of digestate. Typical, and most recent, attempts include stripping [92], chemical precipitation [110], adsorption [111], membrane separation [112], evaporation [113], and electrodialysis [114,115]. The comparison among these approaches is summarized in Table 2.

The concept of integrating nutrient-recovery facilities with AD plants has been widely studied at the lab- or pilot-scale and reported as a promising strategy to contribute to the green transition towards the circular bioeconomy. However, the profitability and sustainability of the full-scale demonstrations for long-term operation are still uncertain and need comprehensive studies to scrutinize their techno-economic and environmental impacts [122]. Fertilizers are currently the main products from digestate recovery facilities, with a market price 80% below their potential value due to the unstable composition, insufficient quality/purity, and weak physical condition [30,145]. In addition, the marketability of fertilizers is dependent on the crops growing season, which could result in additional cost associated with long and expensive storage. Under such circumstances, nutrient recovery from AD slurry for further production of value-added products such as MP can ensure a secure and sustainable market for the upcycled nutrients.

The first criterion for evaluating and comparing the above-mentioned technologies in terms of nutrient recovery from digestate for further MP production is cost-effectiveness. The production cost of MP depends highly on the cost of nutrient recovery and the efficiency of the technology used. When comparing nutrient recovery technologies in terms of operating costs (both chemicals and energy consumption), conventional ammonia stripping and membrane separation seem more cost-effective than other technologies (Table 2). Ammonia stripping (with lime dose) and membrane separation depend less on the consumption of chemicals (keeping in mind that the chemical cost is far more expensive than energy), and pre-treatment is not required for these two approaches. Economic viability is an important reason why these two approaches have been extensively studied and scaled-up. However, they may not be optimal choices when considering maintenance, sustainability, and environmental impacts. For example, although lime is a cheap chemical material used in ammonia stripping, its main problem is fouling of the stripper column by scaling of salts that significantly increases system maintenance [146]. Frequent cleaning of the packing material and using a water-sparged aerocyclone reactor have shown strong evidence in solving scaling and fouling problems. On the other hand, KOH and NaOH are more effective to raise pH but this could lead to much higher chemical costs and alkaline effluent. Even though the purity of the recovered nutrients from these two approaches is independent of the digestate composition, any suspended solids in the liquid fraction will still increase operating costs (e.g., through membrane fouling) [147]. Regarding other approaches, such as ion exchange or chemical precipitation, their promotion is limited by high chemical costs (e.g., for sorbents like zeolites for adsorption and additional doses of Mg^{2+} and PO_4^{3-} ions for precipitation) [13,122].

The second criterion is the accessibility of the recovered nutrients for the proteinaceous microbes. Chemical precipitation is not a suitable method as the recovered nitrogen ends up in a solid struvite, which cannot be easily consumed by either MOB or HOB. Stripping, membrane separation, and electrochemical-driven technologies, which recover the nutrients in the form of ammonia or phosphate salts based on separation and extraction, are available to be employed for MP production. The

high product purity and the physical barrier they provide to potential pathogens and micropollutants are key points which favor using such technologies upstream of an MP production facility [36,115,122]. Membrane separation and electrochemical systems especially can simultaneously recover both nitrogen and phosphorus to support the growth of MP cultures [101,148]. Vacuum/thermal evaporation is not suitable as it cannot isolate and purify ammonia or phosphate from the condensate [140,149]. Ion exchange and adsorption technologies rely highly on the performance of the sorbents and desorbents, without which the product purity cannot be guaranteed [13,122]. Besides which, contaminants (e.g., heavy metals and antibiotics) are difficult to completely remove during the adsorption process. Additionally, disposal of the used adsorption columns could also lead to secondary pollution [150,151].

Electrochemical systems have been employed and tested as an upstream process for MP production [15,19,36]. Table 3 summarizes the proof-of-concept studies for nitrogen recovery and MP production using electrochemical systems. The main merit of the electrochemical approach for nutrient recovery is its potential to be jointly employed with other mature recovery approaches (e.g., ammonia stripping and membrane separation) to overcome the possible technical limitations. For example, when combined with ammonia stripping, electrochemically driven system can save chemical (lime or NaOH) and energy (pumping and heating) costs as the cathodic alkaline environment can be realized via water electrolysis. When combined with membrane separation (compared with traditional RO or UF), the energy for hydraulic or osmotic pressure can be saved and membrane fouling issues could be alleviated, as the suspended solid would not be adherent on the membrane.

A typical electrochemical process (e.g., electrodialysis) for ammonia recovery typically comprises four steps: 1) feeding the digestate into the anode chamber; 2) transferring NH_4^+ ions from the anode chamber to the cathode chamber through cation-exchange membranes driven by an electrical field; 3) accumulating NH_4^+ within the cathode compartment to be converted into NH_3 and OH^- ions (when HOB cultivation is targeted); and 4) extracting NH_3 via stripping [144]. By integrating electrochemical reactors with stripping and membrane technologies, hybrid electrochemical systems can achieve cost-effective nutrient recovery. Meanwhile, product purity, system energy-effectiveness, treatment capacity, and nutrient harvest efficiency should be ensured [101,144]. Moreover, OH^- ions are continuously generated within the cathode compartment due to water electrolysis, saving external chemical addition to maintain an alkaline environment during ammonia stripping and thereby reducing costs [144]. Furthermore, the transport of nutrients across the membrane in step 2 is driven by an electric field instead of mechanical or osmotic pressure, which enhances the recovery efficiency and mitigates membrane fouling compared with conventional membrane separation approaches [148]. In addition, organic micropollutants and heavy metals can be eliminated, and pathogenic microorganisms can be efficiently inactivated with the anodic chamber, which guarantees the stringent hygiene conditions required for MP production [19].

Though promising, the concept of electrochemically recover nitrogen from digestate to MP is still in its infancy. Most attempts made until now have only been at lab scale. Lack of detailed information over upscaled technology limits its commercial exploitation. For successful scaling up of electrochemical technologies challenges still remain to be addressed, including high internal resistance, complicated components (e.g., multi-electrode system, dual- or multi-chamber design), multiple influence factors (applied voltage, membrane performance, electrode with catalyst performance), and difficult online control [69]. Another limitation is the high cost of materials (e.g., electrodes and membrane) [95]. Therefore, development of cost-effective materials and design on high-efficient electrode group structure are needed for technology commercialization.

In general, using digestate for MP production rather than as

Table 2
Comparison of approaches for nutrient recovery from digestate.

Techniques	Main principle	Influencing factors	Advantages	Challenges	Scale
Stripping					
<ul style="list-style-type: none"> • Packed columns [116] • Bubble aeration [117] • Water-sparged aerocyclone [118] • Thermal [119] • Spraying [120] • Vacuum [121] 	Introduce air or steam into stripping towers, converse to digestate flowing: <ol style="list-style-type: none"> i) convert NH_4^+ to free NH_3; ii) diffuse and transfer NH_3 across liquid-interface-gas phase; iii) remove stripping agent [92] 	<ul style="list-style-type: none"> • Temperature • pH • Gas flow rate • Hydraulic loading • Retention time • Packing • CO_2 in the stripping gas • Digestate composition [92,109] 	<ul style="list-style-type: none"> • Low chemical cost (\$1.1–3.5 m^{-3}) • Extensively researched and implemented • Easy operation • High nitrogen load and efficiency (>90%) • Insensitive to solids in the influent stream • Resilient to toxic compounds • Better product quality control [13,92,122] 	<ul style="list-style-type: none"> • Energy-intensive due to pumping and heating ($\sim 4.1 \text{ kWh} \cdot \text{m}^{-3}$) • Air pollution due to NH_3 and SO_2 gas emission • Consumption of chemicals (e.g., lime or NaOH) • Fouling problems due to CaCO_3 scale • High sludge production and high alkalinity effluent • Unable to recover nitrate and phosphorus [92,122] 	Industrial-scale plants, 5–12 m^3 capacity, 500–4500 $\text{Nm}^3 \cdot \text{h}^{-1}$ gas flow, 80–98% efficiency [109,123]
Chemical precipitation					
	<ul style="list-style-type: none"> • Struvite: $\text{Mg}^{2+} + \text{NH}_4^+ + \text{PO}_4^{3-} + 6\text{H}_2\text{O} \rightarrow \text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ • Hydroxyapatite: $5\text{Ca}^{2+} + 3\text{PO}_4^{3-} + \text{OH}^- \rightarrow \text{Ca}_5(\text{OH})(\text{PO}_4)_3$ [101,122] 	<ul style="list-style-type: none"> • pH (8–10.5) • Temperature • Super-saturation • Ion dose and ratio • Agitation • Seeding • Foreign substances [109,124] 	<ul style="list-style-type: none"> • Low energy consumption ($\sim 0.8 \text{ kWh} \cdot \text{m}^{-3}$) • Simultaneous recovery of N and P • Simple operation and construction • Well-proved and good marketability in fertilizer industry [13,109,122] 	<ul style="list-style-type: none"> • Extremely high chemical cost (\$12.9 m^{-3}, dose of Mg^{2+}, NaOH, lime) • Performance limited by the strict ion ratio of Mg^{2+}, NH_4^+, PO_4^{3-} (limited efficiency of 20–65% if without optimization) [125] • Limited variety of end-product application • Residual bittern and high alkalinity in the effluent • Impurity of end-product (hard to isolate solids from digestate) [13,98,109,110,122] 	Full-scale demo, P recovery 80–90%, N recovery 10–40%, crystal/pellet sizes 0.5–5 mm [126,127]
Ion-exchange & adsorption					
<ul style="list-style-type: none"> • Zeolite [128] • Mgo [111] • Resin [129] • Activated hydrochar [130] 	Utilize sorbents filled in a column bed to extract PO_4^{3-} or NH_4^+ from the feed solution, then use desorbent for recovery [13,122]	<ul style="list-style-type: none"> • Temperature • pH • Coexistent ions • Desorption [13,122] 	<ul style="list-style-type: none"> • Simple operation • Low labor cost • Capable of recovering both N and P • Good selectivity and high product purity [13,122] 	<ul style="list-style-type: none"> • High chemical cost (\$8.7 m^{-3}, e.g., column, sorbent, desorbent) • Energy-intensive (2.3–8.9 $\text{kWh} \cdot \text{m}^{-3}$) • Fouling due to suspended particles and precipitates • Competition of foreign ions • Highly dependent on performance of sorbents and desorbents with various recovery efficiencies (43.3–91%) • Reduction of exchange capacity after regeneration • Cannot completely block pathogens, organics and metals [13,109,122] 	Scarce [126]
Membrane separation					
<ul style="list-style-type: none"> • Forward osmosis [131] • Membrane distillation [132] • Ro reverse osmosis [126] • Uf ultra-filtration [133] 	Utilize driving force (hydraulic, osmotic, thermal difference, or external energy) and semipermeable membrane to drive or reject nutrients transferring across the membrane [13,101,122,134]	<ul style="list-style-type: none"> • Membrane property • Fouling and anti-fouling • Solution content • Hydraulic retention time • Pressure • pH [13,122,134] 	<ul style="list-style-type: none"> • Low energy input ($\sim 0.2 \text{ kWh} \cdot \text{m}^{-3}$), excluding RO/UF (6.6–14.4 $\text{kWh} \cdot \text{m}^{-3}$) • Low chemical cost (\$0.7–3.5 m^{-3}) • High selectivity, efficiency (92–99.9%), and product purity • Clean water effluent • Capable of recovering both N and P • Easy to scale-up • Mild conditions • Environmental friendly [13,122,134] 	<ul style="list-style-type: none"> • High maintenance cost due to membrane fouling issues • Contaminant (heavy metals and antibiotics) enrichment • Need reconcentration • Need backwash or regeneration • Low flux and recovery capacity • Pretreatment required to remove particulates and some inhibitors [13,122,134] 	UF, RO, and NF (nano filtration) were applied. 0.5–3.6 $\text{t} \cdot \text{h}^{-1}$ full-scale demo with AD plants, 268–500 $\text{L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ pilot demo, P recovery 80–94%, N recovery 88–98%, 38.20 $\text{gN} \cdot \text{m}^{-2} \cdot \text{mbr} \cdot \text{d}^{-1}$ [135–139]
Vacuum evaporation					
	Create negative pressure conditions and maintain boiling status. Evaporate water and concentrate nutrients [140].	<ul style="list-style-type: none"> • Temperature • pH • TSS • Acid dose [113] 	<ul style="list-style-type: none"> • Low chemical requirement (less alkali dosage) 	<ul style="list-style-type: none"> • Energy-intensive • Low recovery efficiency (18.5–29.9% stripping, 	25–100 L pilot demo, N recovery 97.5%, 5–8 $\text{kWhe} \cdot \text{m}^{-3}$ digestate, 350

(continued on next page)

Table 2 (continued)

Techniques	Main principle	Influencing factors	Advantages	Challenges	Scale
			<ul style="list-style-type: none"> Simple operation and construction Low cost High nutrient load [122,140,141] 	<ul style="list-style-type: none"> 50–70% in condensate) [142,143] Impurity of end-product (TS cannot be removed) Necessary to combine with other methods for recovery Ammonia loss due to volatilization [122,140] 	kWh·m ⁻³ of evaporated water [140]
Electro-chemical system					
<ul style="list-style-type: none"> Electro-dialysis Bioelectro-chemical system 	Electrical-driven ion-exchange membrane system for nutrients' migration, separation, and condensation [141,144]	<ul style="list-style-type: none"> Current pH Electrode material and specific surface area Biocatalyst System resistance [141, 144] 	<ul style="list-style-type: none"> Low chemical input Simultaneous multiple nutrients recovery (NH₄⁺, NO₃⁻, PO₄⁻) High recovery efficiency (~90%) [144] Possibility of neutral energy balance (bacteria activities contribute to energy donation) High product purity Resilience to micropollutants High nutrient enrichment Desalinated water effluent [101,141,144] 	<ul style="list-style-type: none"> Membrane fouling High cost for materials (electrode) and operation Complicated system In-situ pH control is needed Difficulties in full-scale implementation Potentially weak stability during long-term operation (salt precipitation on the cathode causes fouling) [141,144] 	Not yet deployed for commercial applications [144]

Table 3

Recovery of waste nutrient for microbial protein production.

Recovery method	Waste source	Operation mode	TAN gN·L ⁻¹	pH	Voltage V	Current density A·m ⁻²	N removal %	N recovery %	MP	MP DW g·L ⁻¹	Energy consumption kWh·kg ⁻¹ N	Ref
Electro-chemical extract	Synthetic urine	Batch	12	8.05	3.5			35	MOB	0.49	9.97–14.44	[19]
	OFMSW digestate	Batch	2.5	7.3	3.5			33				
	Manure digestate	Batch	2.32	8.1	3.5			50.78				
	Digestate	Batch	0.028						MOB	0.26		[15]
Electro-chemical extract, strip	Digestate	Semi-continuous	0.028							2.32		
	Synthetic urine	Continuous	8.6	9.09	3.05	48	91.6		HOB	4.44		[36]
	Hydrolyzed real urine	Continuous	5.49	9.18	2.66	20	68.4	13.3		1.85 g L ⁻¹	13.9	
	Hydrolyzed real urine + acid	Continuous	5.49	9.18	2.7	20	87.1	13.3		·d ⁻¹	5.8	
Filter, sterilize, assimilate	AD digestate	Batch	0.028				93		MOB	0.255		[14]
	Wastewater	Batch	0.019	7			83		MOB	0.263		[152]

TAN = total ammonium nitrogen; N = nitrogen; MP = microbial protein; DW = dry weight.

biofertilizers would increase its economic value. The product value would increase from around 1 €.kg⁻¹ N for (NH₄)₂SO₄ up to around 17 €.kg⁻¹ N for MP from recovered nitrogen [30]. This process also saves the cost of waste nitrogen removal (600–800 €.ton⁻¹ MP) and brings a profit of around 200 €.ton⁻¹ MP [30,145].

5. Biogas upgrading technologies

MP production is a viable solution to alleviate the world's increasing pressure for protein demand. To achieve a sustainable supply chain for MP production, the development of new strategies, allowing the production of proteinaceous food or feed using renewable energy sources and gaseous streams, should be independent of fossil-based feedstocks (i.e., natural gas). In this context, using biogas produced by anaerobic digestion seems to be an attractive approach, while it offers the possibility to utilize both available carbon sources (i.e., CH₄, CO₂) for MP production [20,41]. In this way, the protein scarcity problem can be

attenuated while environmental degradation is also avoided. Consequently, the combination of AD and MP production contributes to circular bioeconomy.

In this frame, upgraded biogas substitutes natural gas used in 1st generation MP production, reduces the production cost of microbial protein production, and enhances its sustainability. Biogas upgrading technologies, providing carbon and energy sources for MOB and HOB, include (i) water and organic solvent scrubbing, (ii) chemical adsorption, (iii) pressure swing adsorption, (iv) membrane separation, (v) cryogenic separation, and (vi) H₂-based biological methanation technologies [153]. The "best practice" technology for this purpose depends on both the carbon source, which is needed for stimulating the activity of microorganisms, and the aspects related to efficiency, energy demand, and chemical consumption. Comparing biogas upgrading technologies in terms of the above-mentioned factors, will help to find the optimal alternative which matches the concept of 2nd generation MP production, and to establish a sustainable platform for MP production

supporting the vision of circular bioeconomy. The technical specifications of all biogas-upgrading methods are summarized in Table 4.

5.1. Commercialized upgrading technologies

5.1.1. Water scrubbing

The separation of gaseous impurities from biogas using physical absorption, relies on differences in their solubility in the solvent. Having considered water scrubbing as a well-established technology, water plays the role of a selective adsorbent, in which carbon dioxide is up to 26 times more soluble than methane [154]. Methane is also less soluble than hydrogen sulfide (H_2S), resulting in simultaneous removal of H_2S and CO_2 . However, in order to prevent corrosion and operational problems, it is highly suggested to remove H_2S prior to CO_2 , especially when its concentration is high [46].

Depending on the concentrations of non-condensable components of the biogas, i.e., N_2 and O_2 which cannot be excluded, the methane purity can reach 99% [157]. Accordingly, this technology can serve as a promising methane supplier candidate for the stimulation of methanotrophic activity. However, even though water scrubbing has a high pure methane outflow, it cannot deliver pure CO_2 . Nonetheless, when the scrubbing unit is supplemented with a water regeneration unit through air stripping, it is possible to achieve 90% high-purity CO_2 outflow [157], which can be used as a carbon source for HOB. Thus, the regeneration step, besides making water scrubbing a valuable choice not only for MOB but also for HOB, is also a matter of vital importance for ensuring the environmental and economic sustainability of the technology decreasing water consumption greatly, as this technology has a water demand of $\sim 200 \text{ m}^3 \text{ h}^{-1}$ for $1000 \text{ Nm}^3 \cdot \text{h}^{-1}$ biogas [158].

5.1.2. Organic solvent scrubbing

The principle of organic solvent scrubbing is similar to that of water scrubbing except that organic solvents, such as methanol and dimethyl ethers of polyethylene glycol, replace water as the selective absorbent [159]. Selexol® and Genosorb® are the most commercially known selective absorbents which are characterized as being able to dissolve CO_2 and H_2S up to 5 times better than water [158]. Thus, the solvent inputs into the system are reduced and the upgrade unit's dimensions can be smaller, with an associated reduction in investment and operational costs [46,159].

Because of high solubility of the gasses, the regeneration of the solvents is challenging. In case of Selexol®, recovery is even harder and takes place at high temperatures as H_2S is highly soluble in it [46]. Thus, other approaches such as activated carbon filters, adsorption on iron dioxides, precipitation with iron salts or bioscrubbing are proposed for solvent recovery [160] before initiation of the upgrading procedure. Organic solvent-based biogas scrubbing achieves 96%–98% pure methane [161], which can be employed to grow a methanotrophic culture for MP production. Additionally, Sun et al. [157] and Awe et al. [154], reported that high-purity CO_2 can also be generated with this technology. Unfortunately, there is no additional relevant literature to support and verify this option. Thus, further research regarding the potential production of CO_2 is required, in order to form an in-depth opinion on the issue and to assess the eventual utilization of this technology for both MOB and HOB routes.

5.1.3. Chemical absorption

Chemical absorption can deliver biomethane with purity of more than 99% [154,159]. Such performance is a consequence of the use of amine aqueous solutions (i.e., mono-, di- and tri-ethanolamine) and alkaline aqueous solutions (i.e. KOH , $NaOH$, $Fe(OH)_3$, K_2CO_3), which are characterized by high affinity for CO_2 . Thus, methane losses are very low (0–0.2%) [157]. Nonetheless, according to some studies, methane losses above 4% can occur due to its solubility in water, which affects the purity of the CO_2 stream. On the other hand, by means of an air stripper, the resulting rich- CO_2 amine solution can be regenerated, and the

Table 4
Technical specifications of Physicochemical and Biological Biogas Upgrading Technologies [153,154,155,156].

	Water scrubbing	Organic solvent scrubbing	Chemical absorption	PSA	Membrane separation	Cryogenic separation	Sabatier process	Biological upgrading		
								Chemoheterotrophic	Photoautotrophic	Bioelectrochemical
Cost	Medium	Medium	High	Medium	High	High	Medium	High	High	High
CH_4 losses (%)	<2	<2	<0.05	2–4	<0.6	<1	nf ^b	nf	nf	nf
CH_4 recovery (%)	96–99	96–98	>99	96–98	99.5 (MSS) ^a 90–97 (SSS)	97–98	97–99	97–99	97–99	98–99
CO_2 separation	Yes	Possible	Yes	Possible	Yes	Yes	No	No	No	No
H_2S co-removal	Yes	Possible	Contaminant	Possible	Possible	Yes	No	Yes	Yes	nf
O_2 and N_2 co-removal	No	No	No	Possible	Partial	Yes	No	No	No	nf
MOBs	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HOBs	Yes	Possible	Yes	Possible	Yes	Yes	No	No	No	No

^a MSS = Multistage system; SSS = single-stage system.

^b nf = Not found.

entrapped CO₂ with 93% purity can be released [154,162]. Accordingly, chemical absorption technology supplemented with a regeneration unit could be used for the production of pure CO₂ as an alternate product which could find a number of applications, such as for a CO₂ supply to HOB production facilities. In addition, it could be argued that it is a better choice compared to water scrubbing, at least in terms of the CO₂ purity that can be recovered.

5.1.4. Pressure swing adsorption (PSA)

PSA technology separates biogas components with different molecule size, i.e., CH₄, N₂, O₂, and CO₂ by employing adsorbent materials (i.e., zeolite, activated carbon, activated charcoal, silica gel, and synthetic resins) with different affinity for each specific biogas component [163]. PSA takes place in vertical columns, packed with absorbents, and has four distinct phases, namely: absorption, depressurization, desorption, and pressurization [154]. The absorption is performed under high pressure to selectively retain biogas components by the adsorbent material of CO₂, N₂, O₂, H₂O, and H₂S, while methane passes through due to its larger molecule size, resulting in its collection at the top of the column with a decrease in pressure [153]. During desorption, a decrease in pressure occurs, aiming to regenerate the adsorbent material as the trapped gases are released. This gas mixture has to be recycled by returning to the inlet of the PSA process because it contains a considerable amount of methane [159]. The regeneration of the adsorption material requires the separation of H₂S before the start of the upgrading process, as its adsorption is irreversible [46]. PSA has high methane losses up to 4% during desorption, however, can lead to a biomethane purity of approximately 96–98%, and thus its outflow gas can serve as feedstock for the methanotrophic culture [164].

5.1.5. Membrane separation

Membrane-based biogas upgrading technologies benefit from the selective permeability properties of the membranes to separate biogas components. Biogas components have different permeability rates in the following ascending orders: CH₄ (kinetic diameter of 380 p.m.), N₂ (364 p.m.), H₂S (365 p.m.), CO₂ (330 p.m.), and H₂O (265 p.m.) [161]. Diffusion and sorption coefficients are a function of gases' molecular size, resulting in different permeabilities. The smallest molecule, i.e., CO₂, is the least condensable and, therefore, the most permeable [46]. As a result, all gases except CH₄ pass through the membrane, while CH₄ remains in the inlet compartment. This technique is called gas/gas separation and the membranes used are mainly polymeric [162]. However, during this process remarkable losses may occur, since a significant amount of methane (10–15%) is diffused to the permeate side together with CO₂ [46,165]. To enhance CH₄ purity while minimizing its loss, the use of larger size membranes or several membranes in series and recirculation of inlet gasses are recommended. Under these technical configurations, a high methane recovery of 99%, and purity of 95–99% is reportedly achieved [166].

The separation of the biogas components can also be achieved using liquid solutions (i.e., amine solutions) and micro-porous hydrophobic membranes. Practically, the impurities are absorbed by the counterflow of the liquids [167]. The fact that the resultant liquid solution can be regenerated under high temperature, allows the recovery of pure CO₂ [162], which could be possibly utilized for the cultivation of HOBs aiming at the production of high-quality microbial protein.

5.1.6. Cryogenic separation

Cryogenic separation is an emerging biogas upgrading technique, which removes impurities (i.e., H₂O, H₂S, siloxanes, halogens etc.) and CO₂, achieving a biomethane recovery rate of over 97% [167]. Cryogenic separation takes place at high pressures using condensation and distillation processes. This technology relies on different boiling points of methane and carbon dioxide (−160 °C and −78 °C respectively). Thus, after the biogas is compressed to 80 bars, a gradual decrease of temperature down to −110 °C allows for separation of gasses [161]. To

avoid freezing problems, water and H₂S should be removed before cooling [157]. Apart from this, H₂S removal could be considered to be an important step especially if biogas is to be used for MP production, since high content of H₂S can be inhibitory for methanotrophic activity [14], and hydrogenotrophs. The outflow CO₂ has a purity up to 98% [157], marking this technology as a CO₂ supplier for HOB fermentation.

5.1.7. Chemical hydrogenation process

Hydrogenation process of CO₂ can be performed catalytically with the Sabatier reaction, using H₂ as a reductant. According to this reaction, CO₂ reacts with H₂ to produce methane and H₂O with the use of catalysts such as nickel and ruthenium, under elevated temperatures and pressure levels. Although a high recovery rate of CH₄ is achieved (97–99%) [46], chemical hydrogenation has disadvantages compared to biological methods, such as high energy and catalysts demands, as well as sensitivity of the chemical catalyst to impurities in influent gas such as H₂S which can easily toxify the catalyst and hence influence the stability of the process [168]. As a result, even though this process has the advantage of producing a high percentage of CH₄ needed for MP production, its high energy demand and high catalysts requirement undermined its sustainability which urge the future improvements [161].

5.2. Biological technologies

To increase the CH₄ content of off-gases obtained by the anaerobic digestion, biological biogas upgrading can be conducted through chemoautotrophic and photosynthetic reactions. In contrast with physicochemical technologies, biological biogas upgrading takes advantage of beneficial activities of microorganisms and employs them as biocatalysts to valorize CO₂ into CH₄ under mild conditions, e.g., ambient temperature and pressure. The far-reaching benefit of the biological upgrading, however, is that compared to other methods, CO₂ is captured and converted to CH₄ rather than being separated or absorbed [169].

5.2.1. Chemoautotrophic biogas upgrading

The configurations through which chemoautotrophic biogas upgrading may occur with the aid of methanogens, fall into three categories: *in-situ*, *ex-situ*, and hybrid upgrading. Hybrid configuration is a combination of the other two approaches [170], developed to cover the potential problems of each individual system. More specifically, hybrid systems can overcome the intrinsic pH problem occurring in *in-situ* systems, due to endogenic CO₂ consumption. On the other hand, *ex-situ* upgrading chambers employed under hybrid configurations would need a considerably smaller volume since the *in-situ* upgraded biogas is further valorized in the separated reactors [171]. Biological biogas upgrading technologies can reach over 95% CH₄ depending on process performance efficiency and reactor type [46]. Biological biogas seems an interesting approach to provide CH₄ methanotrophs. As the CO₂ content of biogas is also converted into CH₄, instead of being separated and released into the atmosphere without providing any services, the overall energy efficiency is increased [20,51].

5.2.2. Photoautotrophic biogas upgrading (PBU)

Microalgal photosynthesis is another method of biogas upgrading, according to which the CO₂ content of biogas is absorbed by microalgae and a purified CH₄-rich gas leaves the reactors. Besides which, this method is capable of removing contaminants like H₂S, thus improving the calorific value of biomethane [172]. PBU can deliver CH₄ with 97% purity, depending upon the reactor type and the selected algal species [46]. Nonetheless, despite the promising results in terms of biogas upgrading, this method results in excess O₂ concentrations (up to 24%) in the upgraded biogas, which is a major problem due to the gas explosivity. Conversely, in the case of single cell proteins production, O₂ generation could be turned into an advantage, if used in combination with CH₄ for MOB growth [153,173]. Under such circumstances, photosynthetic efficiency is improved, and the use of external oxygen

generator is minimized.

5.2.3. Bioelectrochemical biogas upgrading

A related technology of biological biogas upgrading is combining water electrolysis and CO₂ reduction to methane in a bioelectrochemical systems [169]. In an electrochemical system water splits into H⁺, O₂, and e⁻ in the anode chamber, while in the cathode, the former combines with electrons generating H₂. H₂ is then utilized for microbial transformation of carbon dioxide into biomethane via methanogenesis pathways, which include acetolastic and hydrogenotrophic methanogenesis [169]. Direct electron transfer (DIET) to electromethanogens without the intermediate production of H₂ has also been reported [174]. This mechanism requires that the microorganisms be in direct contact with the electrode and has been reported for members of *Methanosaeta* [175].

Various electrochemical configuration reactors and operating procedures have been reported. In particular, several research groups have scrutinized whether bioelectrochemical systems could be integrated with *in-situ* or *ex-situ* processes and if these designs can include single-chamber systems (membraneless) or more compartments [156]. Electrochemical biogas upgrading can be integrated with MP production. To date, the exploitation of bioelectrochemically upgraded biogas for MP production has only been studied once and its potential has to be validated [41]. In that study, the cultivation of MOB and HOB was combined using cathode off-gas (CH₄ and H₂). The production of CO₂ during MOB's metabolism [20], was directly used by HOBs and therefore a higher protein content and microbial yield were observed [41]. Although bioelectrochemical method is in its infancy, its application as a gas supplier for high-quality MOB-based MP production seems to be a promising approach that needs further investigations.

5.3. Comparing different technologies for coupling biogas upgrading and MP production

When comparing the upgrading technologies in terms of investment and specific costs, effectiveness, consumption of chemicals and energy, it cannot be ignored that the values may change due to supplementary equipment and case-specific requirements. Hence, the best selection should be made with respect to the case in hand and the technology with which they are integrated.

All physicochemical treatment technologies mentioned above, have been demonstrated at capacities ranging from 250 to 3000 m³ h⁻¹ of raw biogas. Considering a plant capacity of up to 2000 m³ h⁻¹ raw biogas, the capital cost for chemical absorption is slightly higher than that of other technologies. However, this investment cost converges with that of other upgrading systems when the capacity and scale get larger. However, for mid-scale range plants, chemical absorption has lower cost compared to membrane biogas upgrading technology or pressure swing absorption [153]. Regarding the marginal cost, it is also influenced by the plant size, and it seems to decrease with an increase of unit capacity. The most economical upgrading technology at a broad scale size (250–3000 m³ raw biogas. h⁻¹) is water scrubbing [153]. The same influence in regard to plant size also applies for marginal costs [157, 158], which vary from 1.50 to 2.32 €/kWh⁻¹ for treatment capacity up to 500 m³ h⁻¹ raw gas and between 0.78 and 1.34 €/kWh⁻¹ for larger upgrading plants [153].

Among all the physicochemical upgrading technologies, membrane and cryogenic separation have higher operating costs. The same applies for chemical absorption, due to solvent cost and loss due to evaporation and the significant energy demand for regeneration [176] (Table 5.1). On the contrary, PSA process is less expensive due to its simplicity, lower energy requirements and capital costs [163].

In contrast to physicochemical technologies, upgrading through CO₂ reaction with H₂ is more expensive due to the high cost of production and storage of H₂ [177]. However, since H₂ can be produced by water electrolysis using renewable electricity (i.e., excess solar and wind

power), whose cost production have been following a decades-long decreasing trend [178], hydrogen assisted upgrading may have a lower CAPEX cost in the future. Given that the price of biomass used for anaerobic digestion may also increase, a further incentive for upgrading biogas by H₂ concerns its possibility of decoupling the biomethanation from biomass availability [153].

If biogas upgrading is coupled with MOB fermentation, the most significant parameter is the purity of methane. All the above-described upgrading technologies can deliver high purity biomethane. Despite that, biological upgrading seems to be more favorable, since it is characterized by the fact that CO₂ is also converted into biomethane [20]. As a consequence, more energy is recovered here compared with physicochemical technologies aiming at CO₂ removal [176]. Nonetheless, any CO₂ remaining in the upgraded biogas (1–3%), could potentially stimulate methanotrophic activity [14]. Referring to the results of Khoshnevisan et al. [20], methanotrophs fed with upgraded biogas had a higher cell dry weight (CDW) yield on methane than those fed with pure methane. Thus, they concluded that this may be attributed to the available carbon dioxide in the gas mixture, which may contribute to aerobic methane metabolism and may be a growth stimulus for some methanotrophs such *Methylococcus capsulatus* [20] and *Methylomonas* sp. Which have the ability to fixate CO₂ [14]. Integration of photoautotrophic biogas upgrading with MP production seems also promising as it stimulates methanotrophic activity and the O₂ can be exploited during MP fermentation [173]. However, photobioreactors need to be further optimized at large-scale operations.

Although CO₂ reduces the calorific value of biomethane, it is a key feedstock for MP production when it is needed in the fermentation process as carbon source. Thereby, the biogas upgrading technology which is selected for biogas plants with downstream HOB processes should be capable of delivering highly purified CO₂. Under such circumstances, water scrubbing, chemical absorption, membrane, and cryogenic separation can be potentially employed as upstream technologies for HOB fermentation. However, the integration of biogas upgrading with MP production needs to be further assessed in terms of sustainability, and techno-economic feasibility, which will help to make the best selection among the available technologies to help direct us toward a sustainable scaling of 2nd generation MP production.

Previous studies of LCA of biogas upgrading technologies showed that the source of electricity, methane losses, and chemicals used in upgrading process are the most important contributors to the environmental profiles of biogas upgrading technologies [179]. The use of low-carbon energy inputs and eliminating methane leakage have been proposed as possible solutions to reduce the carbon footprint of physicochemical biogas upgrading technologies. Since biological upgrading technologies employ excess electricity from renewable sources and utilize microorganisms as biocatalysts for the valorization of CO₂ into biomethane, they will probably be the dominant biogas upgrading technologies in regions with high penetration of renewable electricity production and will contribute to accelerate the sustainable development of MP production. Once the sustainability of the technologies which couple renewable H₂ with biogenic CO₂ is validated for the biogas sector, a wider exploitation can be attempted at industries producing fossil-based CO₂ (e.g., cement, and lime industries) [180]. These CO₂-producing industries can improve their environmental footprint and be more sustainable. Hence, upcycling fossil-based CO₂ to CH₄ (e.g., through biological methanation or Sabatier process) can be also connected with MP production.

6. Supplementary technologies for MP production

6.1. Electrolyzers

Hydrogen is one of the key feedstocks for fermentation in 2nd generation MP. Electrolytic H₂ production, especially using renewable electricity, can contribute to the sustainability of 2nd generation MP.

Accordingly, electrolyzers are an integral part of an MP industry. Electrolysis technologies differ in terms of electrolytes which separate the “oxygen evolution reaction” and “hydrogen evolution reaction”, into anode and cathode, respectively [181]. On this basis, alkaline electrolysis (AEL), proton exchange membrane electrolysis (PEMEL), and solid oxide electrolysis (SOEL) are the main types of electrolyzers [182]. Previously published review papers have comprehensively explained different types of electrolyzers: AEL [183,184], PEMEL [185,186], and SOEL [187,188]. A brief overview over the key characteristics of electrolyzer technologies are given in Fig. 4. Regarding the efficiency of electrolyzers (i.e., cell voltage vs. current density), SOEL has the highest electrical efficiency which can be even higher than 100% if additional heat can be supplied, as shown by Schmidt et al. [189]. However, to produce low-cost 2nd generation MP, the most important factor when selecting the best-suited electrolyser technology is its transient operation (i.e., a state in which the accessibility to electricity is not steady) which are defined by load range, cold and warm start up, and standby losses [190]. This helps to integrate with variable and intermittent renewable electricity sources. Considering these parameters, the load range of AEL is limited to 10%–40%. Otherwise, hydrogen is diffused to the oxygen side across the membrane, resulting in a contaminated, flammable gaseous mixture [183]. On the contrary, hydrogen production in PEMEL operates at full load range (0–100%) and is therefore more compatible with intermittent power grids [191]. Despite the load range of “~100%–100%” available for SOEL, external heat is required to prevent cooling of the stacks which undermines SOEL’s energetic efficiency [192]. Taking other criteria, such as start-up times, into consideration for assessing the electrolyzers compatibility with variable power sources, warm start-up, defined as time interval required to activate electrolyzers, is lower than that of AEL (i.e., a few seconds vs 1–5 min) [190]. Similarly, cold start-up of PEMEL, which is heat-up time required to operate electrolyzers after long shut down at ambient temperature, is much lower than that of AEL (i.e., 5–10 min’ vs 2 h) [191,193]. It should be noted that cold/warm start up time for SOEL is higher if the electrolyzer cannot be held at operating temperature [194]. As a matter of fact, when surplus power is not available, SOEL has a higher standby energy demand compared to AEL and PEMEL, and AEL has higher energy demand than PEMEL. Accordingly, PEMEL will likely be the preferred technology to be integrated with intermittent power supply at standard pressure of 30–50 bar [190,195].

A review of electrolysis technologies indicated that the most prominent and readily commercialized water electrolysis technology is AEL which has a lower cost and higher durability and lifetime compared with other available technologies [186,187]. This is attributed to the absence

of noble metals as well as mature stack components employed in the AEL’s construction [186,187]. However as discussed above, PEMEL, based on the solid polymer electrolyte, can better meet the requirements needed for operating under intermittent renewable electricity sources [196]. Moreover, PEMEL has a lower thermal capacity, lower operating temperature, more compact design, all while producing more purified hydrogen and oxygen [191]. Experts have anticipated that PEMEL would better meet the needs of hydrogen production by utilizing fluctuating power in 2030, hence, this technology would be the most preferred electrolyzer under the global scope [197,198]. However, PEMEL is currently referred to as a less mature technology compared to AEL and it is usually applied for small scale application due to high system complexity, shorter lifetime, and usage of expensive platinum catalyst and fluorinated membrane materials in its construction [189]. Based on the current development of PEMEL technology, 1–2.5 MW electrolyzers have been tested successfully all over the world, including Germany, Denmark, New Zealand, Thailand, and Canada [199]. Many attempts have been performed to upscale PEMEL and reduce its capital costs especially by applying less expensive materials in its manufacturing, improving stack manufacture, and promoting technological learning [197,198]. Accordingly, it is predicted that the cost of PEMEL will be reduced over the next decade as the technology is applied world-wide [189]. Thereby, multi-MW designs of PEMEL were the central projections for the upcoming years. A survey conducted to project the CAPEX (i.e., capital expenditure) of AEL and PEMEL was carried out regarding their historical CAPEXs [200]. According to the results, the CAPEX of PEMEL could compete with that of AEL in 2030 and its CAPEX was projected to reach 700 €/kW_{el} in 2030.

6.2. Carbon dioxide capture technology

CO₂ capture technologies also have a high potential for being integrated with the MP industry as they can supply purified carbon dioxide needed for the fermentation of HOB. This integration will be more prominent when the produced biogas is intended for energy purposes, and biological biogas upgrading is employed to valorize the CO₂ content of biogas into biomethane [29,201]. Several CO₂ capture technologies have been developed to separate CO₂ either from different industrial exhaust gases (e.g., flue gas, biogas, natural gas, kiln gas and landfill gas) or directly from air known as direct air capture (DAC), typically including CO₂ absorption, adsorption, and membrane-based extraction [202–206]. Compared to CO₂ capture from the concentrated industrial exhaust gases that has been extensively studied and achieved commercial application in recent years, DAC is normally operated under an

Fig. 4. An overview of main features of three main types of electrolyzer technologies. Data were taken from Refs. [183–185,187–190].

Advantages of membrane technologies include no chemicals/solvents requirement, simplicity of operation and maintenance, and process flexibility. Disadvantages are the low CO₂ purity output, high investment cost, and unstable long-term operation. This technology has been successfully applied to capture CO₂ from various industrial streams, such as flue gas (12–15 vol% CO₂) [206]. However, there are few reports using this technology for DAC (~0.04 vol% CO₂ in air) [206].

7. Fermentation and post-processing of MP

Gas transfer to the water phase is the main factor limiting the production of MP given the low solubility of both methane and hydrogen [46]. Thus, the design and operation of bioreactors aim at maximizing mass transfer [57,232,233], thereby maximizing bacterial biomass productivity.

For the case of methanotrophs, continuously stirred tank reactors (CSTRs) have been extensively used for the continuous fermentation of MP. They provide homogeneous concentrations within the bulk liquid and maximize the contact of cells with nutrients and dissolved gases [232]. MP fermentation at lab scale mostly cultivated in serum bottles, one third of which was the cultivation media, and the headspace was a mixture of oxygen and methane [19,20,41]. While this set-up is useful for fast screening and optimization of different cultivation conditions and feedstocks [15,20,34,38,234,235], they have provided low gas-liquid mass transfer, and hence are not suitable for large scale biomass production. To deal with gas-liquid transfer limitations in MP fermentation, different gas diffuser technologies have been proposed. *M. capsulatus* has been grown in batch fermenters supplemented with ceramic stone diffuser, treating wastewater from a potato processing plant [152]. Such set-up could achieve a fast specific growth rate of 4.35 d⁻¹ and biomass concentration of 0.26 g-TSS. L⁻¹, but faced limited methane and nutrients uptake, likely due to the lack of micro-nutrients in the cultivation media. Tsapekos et al. [15,34] used a similar fermenter configuration for cultivating an enriched-mixed methanotrophic culture on digestate from urban biowaste but employed “Marprene Tubes” for gas supply. In their first study [34], they achieved biomass

concentrations ranging from 0.6 g-VSS. g-L⁻¹ at 4 days solid retention time (SRT) to 0.29 g-VSS. L⁻¹ at 2 days SRT. Increasing the SRTs did not have a significant effect on protein content and therefore decreased the MP productivity of the bioreactor [15]. However, they could enhance the biomass productivity by supplementing the same feedstock with 100 µgCu²⁺.L⁻¹, augmenting production by more than 40%. The inclusion of impellers can further enhance mass transfer in the fermentation broth [232], but they are not recommended because methanotrophs suffer from mechanical stress. In 1 L fermenters, biomass productivity was halved when the rotational speed of a “Rushton Turbine” increased from 300 to 600 rpm, shifting from gas transfer limitation to microbial activity limitation [236]. With a rotation speed of 200–300 rpm, a methanotrophic enrichment could achieve a cell density of 2.69 g L⁻¹ during a 25 h batch test [39]. The selection of diffuser is also important in this context. The use of hollow fiber membranes with small pore size and high contact area can enhance mass transfer compared to traditional alumina bubbler. Hollow fiber membranes could increase gas transfer by 262% compared to alumina bubblers, which yielded an increase of 67% in growth rate and 77% in biomass concentration [237].

Forced flow has been proposed as an alternative to diffusers to build up a high efficient bioreactor with sufficient methane supply [238]. First designs, however, had low gas transfer coefficients (k_La), ranging between 120 and 545 h⁻¹ [239]. The U-loop fermenter was patented in 2000 [240] and is currently exploited at commercial scale by Unibio® S/A. In brief, gas and liquid are mixed by pumping them through a vertical U-shaped pipe fitted with static mixers (Fig. 6). Static mixers enhance the gas mixing as well as avoid bubble sticking to the reactor walls. The U-shaped pipe leads to a cylindrical top de-gassing tank. The gas phase has a short holding time (<1 min), versus several hours for the liquid phase, and behaves as a near plug flow reactor. The liquid phase is radially mixed achieving high gas mass transfer, and its overall residence time in the U-loop reactor determines the biomass productivity [241]. This reactor configuration can achieve k_La ranging from 1200 to 3000 h⁻¹ and biomass concentrations up to 30 g L⁻¹ [232]. Nevertheless, they have never been applied with residual streams, whose viscosity and composition may affect the mass transfer.

Fig. 6. (A) Technical drawing of a U-loop reactor. Locations of sensors and feed inlet positions are marked with a dot or an arrow respectively. Offline measurements are shown in a dashed box. (B) Diagram of the liquid flow in the U-loop reactor (Reproduced from Ref. [242] with permission Elsevier Copyright (2021)).

Similar to methanotrophs, hydrogenotrophs are often cultivated in serum bottles in the lab-scale experiments, which allows for fast screening of operational conditions [243–245]. Table 5 compares bioreactor performances for MOB and HOB cultivation. Classical CSTRs with bubbling systems and impellers to improve mixing have been introduced as the preferred bioreactor for the cultivation of HOB. Matassa and colleagues [26] used a CSTR in batch mode, reaching a productivity of 0.078 g-VSS. L⁻¹h⁻¹ with a mixed culture dominated by *Bdellovibrio* spp. Surprisingly, when shifting to continuous flow operation with a dilution rate of 0.1 h⁻¹, the productivity increased up to 0.375 g-VSS. L⁻¹h⁻¹ and the culture enriched in *Sulfuricurvum* spp, reaching a protein content of 71% CDW. Following a similar approach, another study reached a similar yield on hydrogen using a mixed hydrogenotrophic culture fed with nitrates instead of ammonia [246]. In this case, when applying a dilution rate of 0.1 h⁻¹ the culture was dominated by *Azospirillum lipoferum* strain DSM 1691. However, this study focused on biomass application as biofertilizer, and microbial protein content was not reported. Despite the promising results, hydrogen oxidizing bacteria are not exploited at full scale yet and further development is needed to reach commercialization.

Despite the promising results, fermenters relying on bubbling for gas supply may suffer from explosion risks, as methane and hydrogen have an explosion limit of 5% [247] and 4% [248], respectively. Thus, even mass transfer may be efficient, operation of the bioreactor stops whenever the composition of the gas phase approaches the lower explosive limits, hampering optimal biomass productivity. To avoid risk of explosion, methanotrophs can be co-cultivated with green microalgae [249–252]. Microalgae release in-situ oxygen, which becomes available for aerobic methane oxidation by methanotrophs, which produce carbon dioxide. Then, carbon dioxide becomes available for algal growth. When co-cultivating *Chlorella sorokiniana* with *M. capsulatus* in a batch CSTR, the biomass concentration reached 1.5 g-VSS. L⁻¹ while the cultivation of *M. capsulatus* alone reached 0.26 g-VSS. L⁻¹ [152]. However, the protein content of the co-culture was lower than the methanotroph, while the lipid content was higher. Furthermore, oxygen accumulated in the gas phase reached explosive levels. It was hypothesized that the competition for some of the trace elements prevented optimal growth of both microbes. For example, competition for metals such as copper, which can easily bioaccumulate in green microalgae [253], could decrease methanotrophic activity and prevent oxygen uptake. Other aspects such as light intensity can be controlled to modulate photosynthetic activity, thereby releasing oxygen at a speed that methanotrophs can take it up. It is also important to avoid excessive oxygen production, which could potentially reach inhibitory levels. Robert and colleagues [252] used the same consortium to valorize wastewater and digestate from a municipal wastewater treatment plant. They found better

performance by the co-culture, reaching biomass concentrations of 3 g L⁻¹ and about 80% TN recovery. However, protein content was only 40% of the total dry weight, which is below the typical content for methanotrophs used in the feed industry. Interestingly, the oxygen level during the cultivation raised towards the end, when most methane was already consumed, minimizing the risk of explosion. Raw biogas was used to feed a co-culture of *M. alcaliphilum* and cyanobacteria, *Synechococcus 7002*, in synthetic cultivation media [254]. They observed improved biomass productivity compared to the mono-cultivation of a methanotroph. Interestingly, the co-culture was not inhibited by the presence of H₂S, which has a detrimental effect on methanotrophic biomass [34,43]. This co-cultivation strategy cannot be applied with hydrogenotrophic bacteria, as they compete with microalgae for carbon dioxide [255].

Another approach to avoid explosive gas mixture is the use of hydrophobic membranes for gas diffusion, in the so-called membrane aerated bioreactors (MABRs). These reactors are widely used in wastewater treatment for efficient aeration [256], but also for the cultivation of anaerobic methane oxidizers as biofilms intended for nitrogen removal [257]. A bubble free MABR with two different cassettes of hollow fiber membranes was designed and optimized to supply methane and oxygen by diffusion to the liquid phase, without creating explosive atmospheres [258]. Having operated at similar conditions with a fermenter supplemented with bubbling gas suppliers (i.e., same dilution rates and bacterial cultures), the bubble free MABR had lower biomass yields of 0.43 g-VSS. g-CH₄⁻¹ vs 0.79 g-VSS. g-CH₄⁻¹ at 4 days SRT [34]. The maximum biomass density reached during the operation of the reactor was 0.25 g L⁻¹. However, more protein was stored in the biomass, making it more suited as feed ingredient. Authors suggested that reactor design could be improved by increasing the membrane area to reactor volume ratio in flow through membrane systems or feeding rates via dead-end membranes, which can potentially increase biomass productivity. Interestingly, by controlling the oxygen to methane v/v ratio biofouling could be minimized and bacteria grew mostly in suspension.

Similar approach can be applied for hydrogenotrophic fermenters, where hydrogen and oxygen could be diffused to the cultivation media without creating explosive gas mixtures. Indeed, hydrophobic membranes have been already used for biological biogas upgrading [259] or hydrogenotrophic denitrification in anoxic environments [260], but they have not been applied for microbial protein production yet. In bioelectrochemical reactors, cathodes are used to produce hydrogen gas via water electrolysis. H₂ is produced on the cathode surface, which can be taken up on biofilms developed onto the cathode before reaching the gas phase [261]. However, the presence of oxygen hinders the production of hydrogen and therefore thick biofilms are desired, allowing for

Table 5

Examples of bioreactors used for microbial protein production and their operational parameters.

Microbial Protein	Max. Yield on CH ₄ or H ₂	Max. Cell density (g.L ⁻¹)	Max. Volumetric biomass productivity (g.m ⁻³ .d ⁻¹)	Max. Protein content (% of CDW)	Species	Reactor type and operation	Reference
MOB	0.43 g-CDW.g ⁻¹ .CH ₄	0.25	59.54	51	Enrichment <i>Methylobacter</i> sp and <i>Methylocystis</i> sp	Membrane aerated continuous stirred bioreactor in continuous flow	[42]
MOB	0.79 g-CDW.g ⁻¹ .CH ₄	0.95	120	41	Enrichment <i>Methylobacter</i> sp	Continuous stirred tank aerated with marprene tubes in continuous flow	[34]
MOB	0.37 g-CDW.g ⁻¹ .CH ₄	2.5	464	53	Enrichment <i>Methylobacter</i> sp	Continuous stirred tank aerated with marprene tubes in semi-continuous flow	[15]
HOB	0.28 g-CDW.g ⁻¹ .COD	4.44	9000	71	Enrichment <i>Sulfuricurvum</i> sp	Continuous stirred tank aerated with micro spargers in continuous flow	[26]
HOB	0.18 g-CDW.g ⁻¹ .COD	0.13	312	n.r.	Enrichment <i>Azospirillum</i> sp	Continuous stirred tank in continuous flow	[246]

n.r.: Not reported.

gradients within the biofilm. Thereby, oxygen could be provided from the liquid phase and depleted before reaching the cathode surface, without compromising the water electrolysis. However, in order to have high volumetric production rates high bio-cathode surface to reactor volume ratios are needed, which will likely hamper the harvesting of the biofilm. A bioreactor coupled with a water splitter was proposed to solve these issues. Water and microbes flowed through an external stack of electrodes, where water electrolysis took place. Most growth took place in the bioreactor, where the CO₂ was supplied. The combined system was demonstrated to be more efficient than direct immersion of the electrodes in the fermenter [262]. Still in most cases water is typically split in electrochemical cells and the off-gas hydrogen is fed to the fermenters where, it is mixed with CO₂ and the oxygen provided by the anode and the hydrogenotrophs grow in suspension [263]. This indicates that separate water electrolysis in an electrolyser, which can efficiently split the water to H₂ and O₂ and subsequent utilization of the H₂ generated in a separate reactor, is to be preferred.

Fed-batch is the most common cultivation strategy for MP production [232]. Following this approach, high biomass concentrations at the end of the cultivation are reached, which increases the cost efficiency of the harvesting process. Since most studies are conducted at laboratory scale, the standard harvesting method consists of centrifugation and freeze-drying of the up-concentrated biomass [42] or direct drying in ovens [264]. This is also the approach followed at full-scale application, where the culture broth is first centrifuged to a high biomass density paste, which then typically undergoes a spray-drying process [265]. Despite this approach is industrially used, these processes are energy intensive and often compromise the feasibility and sustainability of the MP value chain. Forward osmosis (FO) has been proposed as a less energy intensive biomass thickening method [38]. FO relies on an osmotic pressure gradient as driving force to dewater the culture broth and therefore the main cost relates to pumping the flow through the membrane module. In a recent study, FO was evaluated for the up-concentration of methanotrophic biomass during 48 h batch experiments [38]. The authors proposed the use of residual brine from desalination plants, so no reverse osmosis was needed to recover the water. Besides, brine is currently a residue that needs treatment solutions before being discharged to the sea. FO achieved high concentration factors (1.5–2) and detected short-term biofouling did not affect the overall performance. However, longer-term evaluation is still needed to demonstrate that this technology can outperform pressure driven filtration. Finally, given the methanotrophs and hydrogenotrophs propensity to grow as biofilms, engineering of advanced membrane biofilm reactors can be a solution to minimize the explosion risk, achieve high-rate gas transfer and reduce harvesting costs. For microalgae, it has been already demonstrated that biofilms have high cell density and are easy to recover by simple scraping or vacuuming [41].

8. Sustainability aspects

Global food production entails serious environmental damages [266]. Protein production as part of the global food supply chain accounts for a considerable fraction of the impacts imposed [267]. As is evident, protein production practices should be modified to meet sustainability requirements. This can be achieved by identifying alternate protein production pathways, which can adapt to future needs and mitigate environmental impacts from the global food supply chain. In this context, in-vitro meat [268], insects [269], or MPs [17] have been introduced as well-suited alternatives for reducing impacts across the food system. Herein, environmental, economic, and circularity aspects of MPs via MOB and HOB routes are reconsidered to scrutinize the current challenges and suggest future improvements.

Due to growing interest towards the use of MP as feed supplements for livestock and aquaculture, their environmental impacts, compared to other protein sources, have been recently studied by few researchers. Having employed “MAGPIE 2” model, Pikaar et al. [201] studied the

impacts of different MP production pathways assuming that MPs replace conventional protein feed sources. The scenarios included the use of nitrogen from Haber-Bosch process and carbon source from sugarcane-to-MP, Biogas-to-MP through AD of maize, Hydrogen-to-MP through electrolytic hydrogen production and exploitable carbon dioxide, syngas-to-MP through gasification of *Miscanthus* spp., and natural gas-to-protein. They showed that it is possible to gain benefits regarding the land use, water use, and climate impacts, when producing MP from various pathways. The most reduction potential was shown to be a pathway using HOB produced via water electrolysis and renewable energy with an avoided cropland expansion of 109 million-hectare, 40 Gt of avoided CO₂ equivalents, and 10 Tg of avoided nitrogen pollution. The lowest environmental benefits were achieved from biogas-to-MP (MOB) pathway, where biomethane was produced from forage crops. Relevant pathway for this study, is also syngas-to-MP (HOB) showing the second largest reduction potential in the studied systems. In addition, Khoshnevisan et al. [7] performed a consequential LCA for two MOB production pathways, where biogas slurry from anaerobic digestion of household waste was used as culture media while biogas and biologically upgraded biogas were utilized as carbon source. Both pathways showed reduction potentials regarding climate change and resource use. Based on these studies, both HOB and MOB as MP can result in major net-positive environmental benefits. However, to understand the key unit processes affecting the impacts, further research is needed.

The 1st generation MP using MOB has already been commercialized and the environmental impact of the scaled-up production has been investigated [44]. However, 2nd generation MP using MOB pathways has not been scaled-up and thus the environmental impacts at industrial scale have not been scrutinized so far. Furthermore, only limited studies have investigated HOB-based 2nd generation MP at pilot scale from LCA points of view [22,29]. The literature review shows the lack of deep investigation on sustainability aspects of both MOB and HOB production facilities, especially under the integrated system with anaerobic digestion plants and the associated nutrient recovery and biogas processing facilities.

Having compared 2nd generation HOB and 1st generation MOB production, HOB route using renewables showed lower impacts than MOB route, even if MOB production facility substituted natural gas with biogas [22,29,44]. As shown by Sillman et al. [29], HOB production pathway under the best-case scenario, which uses wind or solar energy, could have 53% and 42% lower global warming potential (GWP) than MOB pathway using renewables and biogas, respectively. Having considered their water footprint, the blue water consumption of HOB routes could be considerably lower than that of MOB routes while land use (LU) impact was found to be similar. It is worth noting that up to 80% of cultivation medium can be circulated within the fermentation process which considerably decreases the water footprint of MP production. In this context, water electrolysis which is used for hydrolytic hydrogen production has small contribute the overall water footprint of MP production (Supplementary information SI-3). Having included the water consumption of electrolyzer facilities coupled with biological biogas upgrading [51], MOB-based MP production requires up to 137 L water.kg⁻¹ pure protein depending on the waste effluents and opted pretreatment method [33]. Hydrogen production utilizes 3.5–8.1 L water.kg⁻¹ of pure protein (Supplementary information SI-3). Sillman et al. showed that the production of HOB-based MP requires 3.8 L water.kg⁻¹ protein [29]. Järviö et al. used water scarcity to investigate the impact of water use in HOB-based MP production. They showed water scarcity had a high uncertainty and ranged between 0.14 and 0.37 m³ kg⁻¹ final product with 65% protein. Moreover, they concluded that when electricity has a hydropower origin, water scarcity of MP sharply increases [22]. The water consumption of FeedKind protein, i.e., commercialized 1st generation MP, is reportedly 10–29 L water.kg⁻¹ protein [44]. As can be seen, the water footprint of MP still suffers a high uncertainty and future research is needed to close the gap.

Järviö et al. [22] studied the environmental impacts of a HOB-based pilot-scale facility using renewable energy without state-of-the-art technologies. They reported 8% lower GWP impact from HOB pathway compared with MOB production based on renewables. The key unit process affecting the environmental sustainability was reported to be water electrolysis contributing to 90% of the total energy demand of HOB production. Another process with significant contribution to environmental profile was ammonia consumption derived from Haber-Bosch, accounting for approximately 64–90% of the total GWP of best-case scenarios. The utilization of nitrogen nutrient from side-streams can considerably lower different environmental impacts compared to conventional Haber-Bosch technology [270,271]. Thus, nitrogen recycling according to the pathway shown in Fig. 2-A can even further increase the environmental benefits of HOB compared to 1st generation MOB, when renewables are used as the main energy source.

HOB protein production using water electrolysis is an energy intensive process, thus the source of energy for hydrolytic H_2 production considerably affects the environmental performance and production costs [16,30]. The same applies when biologically upgraded biogas is used for 2nd generation MOB production, where water electrolysis is used to provide hydrogen for methane formation [46]. There are other benefits than low GWP, LU, and freshwater use (FWU) related impacts.

when producing MP via proposed approaches (Fig. 7): i) MP production facilities can be located in non-arable land areas; ii) the production processes can be designed as a closed system without nutrient runoffs; iii) the production does not require herbicides or pesticides which have negative impacts on biodiversity; and iv) the production process is controllable [45]. In addition, the possibility to use nutrients from various sources, such as from wastewaters and manure, makes the MP production an excellent solution as part of circular bioeconomy [17,30].

Upstream technologies play a key role in the sustainability aspects of MP production. Nutrient recovery technologies employed to extract nutrient from waste effluents comprise an important stage of 2nd generation MP. Shi et al. [13] reviewed different nutrient recovery technologies developed for nutrient recovery from anaerobic digestate of manure. They reported that ammonia stripping and struvite formation would have much easier operation than other technologies considered. Membrane technologies showed promising results in this context if membrane fouling can be effectively dealt with. A recent study has ranked five different nutrient recovery technologies (i.e., air stripping, ion exchange, struvite precipitation, reverse osmosis, and gas permeable membrane separation) considering several criteria including economic and recovery performance. Having considered the aforementioned criteria, permeable membrane separation has been nominated as the

Fig. 7. Circularity and benefits of the proposed MP production pathways.

best technology followed by struvite precipitation [272]. Having considered the avoided impacts from synthetic nutrient production, many nutrient recovery technologies have reportedly resulted in lower environmental burdens [271,273]. Thus, nutrient recovery technologies can improve the environmental sustainability of HOB and MOB production. However, net positive GWP seems not to be possible with nutrient recovery technologies [273].

Ardolino et al. [274] reviewed and assessed several most utilized biogas-to-biomethane production pathways, which are membrane separation, water scrubbing, chemical absorption with amine solvent and pressure swing adsorption. From environmental and economic points of view, the highest net-positive impacts and lowest costs can be gained by using membrane separation technique, but the difference among the studied techniques are not conclusive. However, net-positivity was gained when considering the avoided impacts by substituting fossil fuels with biomethane. Hence, the amount of environmental impacts of biogas-to-biomethane is highly dependent on how the biomethane is utilized [4,5]. If the biomethane is all used to produce MOB-based MP, the avoided impacts through product substitution of biomethane becomes questionable. Then again, if the separated CO₂ after biogas-to-biomethane is used to produce HOB-based MP and the biomethane is partly used as an energy source and partly as C source for MOB-based MP production, the net-positive impacts of biogas upgrading process can be possible. However, future research is still needed in this context to comprehensively consider all the possible routes from the sustainability aspects.

The environmental sustainability of 2nd generation MP is dependent on how the ongoing renewable energy transition goes. If the world shifts to rely on renewables, such as solar photovoltaics (PV) and wind turbines, they cause fluctuations in the grid, which need to be balanced. Different smart grid solutions with goals to integrate customers have been proposed to overcome this challenge [275]. One interesting solution is to use power-to-gas (PtG) applications, which can be used as a demand response solution to provide gas, such as methane, for energy storages and other uses. The gas can be used to balance the grid by producing energy via gas turbines, when needed. These PtG solutions are feasible in economic and environmental aspects in future energy systems [276–278]. Hence, using biological biogas upgrading, a PtG application, to provide methane for energy purposes becomes an interesting option (Fig. 2-C).

The production cost of 1st generation MOB-MP production consists of operational and capital expenditure of AD, nutrient recovery, MP fermentation, dewatering and biomass drying varied between 920 and 2530 € per ton of MP [30]. According to their results, 71% of the total costs were related to MOB growth, from which 46% of the total cost accounted for AD process while ammonia recovery and fermentation constituted 20% and 19% of the overall cost, respectively. The results showed that the future economy of 2nd generation MOB-MP will depend on technological improvements and optimal design of the platform for decreasing the production costs. Verbeeck et al. [30] demonstrated that 2nd generation HOB-MP production was less economically attractive than 1st generation MOB-MP production at the current production prices of renewable hydrogen. According to their results the production cost varied between 1590 and 3780 € per ton of MP with 67% contribution from hydrogen production facility. However, Verbeeck et al. [30] considered higher hydrogen need for HOB production than the real values reported [22,279,280]. Thus, there would be a possibility to decrease the cost of hydrogen production. However, with the current electricity prices, the more feasible production pathway would be 1st generation MOB-MP route, when considering economic and environmental sustainability of different pathways.

When considering the future economic aspects of the studied pathways in high renewable energy systems, it is possible that biological biogas upgrading costs becomes more cost-efficient than CO₂ removal from biogas due to possible technological development and rapid cost decrease of renewables [46,51]. In this case, the production pathway C,

presented in Fig. 2 and Table 6, might become the most economically feasible MP production pathway. On the other hand, the cost reduction also applies for pathway A, so it cannot be said for certain which of these two pathways, A or C, would be economically most feasible in future. Still, the energy demand for pathway A would be higher than for pathway C as the A requires more H₂ than pathway C based on process stoichiometry. Whether to separate CO₂ from biogas for HOB production and use the excess methane for energy purposes or to provide methane for MOB production and/or for energy purposes, depends on food and energy systems. Both pathways provide a sustainable solution for a different challenge. Thus, techno-economic-environmental analysis considering the energy and food systems should be conducted in these cases (e.g., Table 6). When considering the feasibility of combined HOB and MOB production pathway, the combination can maximize the process efficiency regarding the available material flows from biogas, and it has been tested as a proof-of-concept level [41]. To be feasible alternative, similar kind of technological development should happen than for A and C pathways. However, the combined technology would increase the investment and operational costs as both MOB and HOB MP production technologies would be required. From product based environmental aspect, the combined MP production pathway would fit somewhere between A and C, as it uses similar technologies.

9. Conclusion and prospective work

MP, i.e., a land independent protein source for livestock and

Table 6

An example of production pathway comparison based on economic and environmental sustainability feasibilities. A, B, and C refer to the pathways shown in Fig. 2.

	HOB (A)	MOB (B)	MOB (C)
Low renewable penetration			
Economic feasibility	LOW (High investment and operational costs)	MODERATE (Moderate investment and operational costs)	LOW (High investment and operational costs)
Environmental feasibility	LOW (High greenhouse gas emissions compared to production pathway B due to electricity consumption)	HIGH (Low greenhouse gas emissions compared to production pathways A and C due to small electricity consumption)	LOW (High greenhouse gas emissions compared to production pathway B due to electricity consumption)
High renewable penetration			
Energy system requiring extra gas for grid balancing			
Economic feasibility	MODERATE/HIGH (High investment and low operational costs)	MODERATE (Moderate investment and operational costs)	MODERATE/HIGH (High investment and low operational costs)
Environmental feasibility	HIGH (Low impacts due to renewable electricity consumption and production of biogas and MP)	MODERATE (Moderate impacts compared to production pathways A and C)	HIGH (Low impacts due to renewable electricity consumption and production of biogas and MP)
Food system requiring sustainable protein source			
Economic feasibility	MODERATE/HIGH (High investment and low operational costs)	MODERATE (Moderate impacts compared to production pathways A and C)	MODERATE/HIGH (High investment and low operational costs)
Environmental feasibility	HIGH (Low impacts due to renewable electricity consumption and production of MP)	MODERATE (Moderate impacts compared to production pathways A and C)	HIGH (Low impacts due to renewable electricity consumption and production of MP)

aquaculture, has provoked considerable interest among researchers and the first generation of MP, grown on natural gas and synthetic chemicals, has been already commercialized. Researchers have been attempting to shift from 1st generation MP to 2nd generation by substituting the nutrients extracted from waste streams for synthetic chemicals and replacing natural gas with biomethane. A wide range of technologies, i.e., anaerobic digestion, biogas upgrading technology, nutrient recovery, fermentation, and post-processing technologies, should harmonically work at upstream and downstream to support sustainable production of 2nd generation MP.

Nutrient recovery from waste effluents such biogas slurry is the heart of 2nd MP supply chain, because to a great extent it determines the overall production costs and the safety of the final product. Electrochemical/bio-electrochemical systems combined with stripping and membrane systems will have a higher chance to be the dominant technology at upstream, on the one hand, due to higher efficiency and lower operational costs, and on the other hand, because of safety and quality of the final product. However, the technology is still immature and needs further research, especially needs scaled-up demonstration. Furthermore, nutrients from other waste effluents than digestate, such as municipal wastewater and bakery wastewater should be targeted to increase accessibility to nutrients for microbial protein production.

The environmental sustainability of 2nd generation MP is dependent on how the ongoing renewable energy transition goes. In regions with high penetration of renewable energy and occurrence of off-peak surplus electricity production, MOB-based MP production route with biological biogas upgrading at downstream can be a sustainable alternative. Biological biogas upgrading uses surplus renewable electricity at lower price and upcycles CO₂ content of biogas into CH₄ which can be partly used by microorganisms as energy and carbon sources while the rest can be stored in the gas pipelines at low storage price. Wherever biological biogas upgrading is not probable, or carbon capture technologies (especially DAC) are available to deliver purified CO₂, HOB-based MP production can be scaled up as the future technology to substitute conventional protein sources. However, regardless of the possible routes for HOB and MOB-based MP production, fermentation technology is also a determinant factor herein.

Gas transfer to water phase is the key challenge to which MP fermentation faces. Such bottleneck making conventional fermenters such as CSTRs inefficient for achieving a high productivity and biomass yield. Moreover, using conventional fermenters for MP production increases the HRT, and the total fermenters' capacity needed, hence, raises the capital expenditures associated with MP production facility and in most cases undermines the economic feasibility of the process. U-loop reactors with forced flow concept has a gas transfer coefficient as high as 3000 h⁻¹ which enables reaching a biomass concentration of up to 30 g L⁻¹. Besides the fermentation technology, 2nd generation MP production still seeks some biotechnological inventions and metabolic engineering to facilitate MOB and HOB cultivation with high N to protein conversion rate, less sensitivity to microbial growth stresses, and higher amino acid profile comparable with conventional protein sources.

This study would have some practical implications towards food safety and inadequate protein resources. Currently, making waste into high quality food/feed is accredited as an environmentally friendly solution and a cost-effective business. Among these, the production of 2nd generation microbial protein has shown great potential as a nutrient supplier for both humans and livestock. The information obtained also is commodious for consumers and commercializing such products and changing government policies. Though promising, several challenges are still needed to be overcome before a broad implementation. First, MP, as a new protein alternative, is currently not widely accepted by the market as animal feed or human food. It is still unknown whether farmers are willing to use 2nd generation MP instead of traditional crop-based animal feed. Thus, extensive promotion and official legal recognition are necessary to receive a widespread public acceptance of microbial-derived proteins, with an emphasis on food sustainability and

low environmental impact. Second, the marketability and profitability of the technologies for "digestate-to-MP" could be challenged by the sophisticated facilities/mechanisms, high investment, premature technology, and complicated operation/maintenance. Thus, more pilot-scale demonstrations and thorough techno-economic/life cycle assessment are needed before full-scale application. Third, more measures are required to guarantee the safety and quality of MP, especially when the nutrients are derived from organic waste like AD digestate. Requisite sterilization process and physical barriers such as membrane should be considered to prevent the cross-over of harmful contaminants.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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