Enhancing methane production in anaerobic digestion through hydrogen assisted pathways – A state-of-the-art review

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Abstract

Anaerobic digestion has been widely accepted for energy and resource recovery from biomass residues. However, the produced biogas from the process mainly composed of methane and carbon dioxide is lower in calorific content, which is a major drawback for its direct application as an energy fuel. Therefore, different biogas upgradation systems based on physical, chemical, and biological processes have been applied to remove carbon dioxide and other gaseous constituents from the biogas and utilize carbon dioxide into methane. This review discusses the possible hydrogen-assisted pathways for converting carbon dioxide into methane in the presence of hydrogen and improving its proportion in the biogas composition during anaerobic digestion through *in-situ* biogas upgradation. Additionally, a co-production of hydrogen and methane in two-stage anaerobic digestion has been proposed for methane enrichment. Technical challenges, stabilization of process parameters, innovative modification and microbial pathways have been explored and discussed. The findings and prospects from this article could be an interesting state-of-art for optimizing process parameters during hydrogen-assisted pathways and its mainstream application on existing digestion systems.

Highlights

- *In-situ* microbial methane enrichment technique through hydrogen assistance is reviewed.
- Organics rich substrates are suitable for hydrogen assisted microbial methane enrichment.
- Hydrogenotrophic methanogenesis and homoacetogenesis are the key pathways involved.
- Modified two-stage anaerobic digestion for microbial methane enrichment is proposed.

Keywords: Anaerobic digestion, Hydrogenotrophic methanogenesis, In-situ methane enrichment, Microbial interactions, Wood-Ljungdahl pathway

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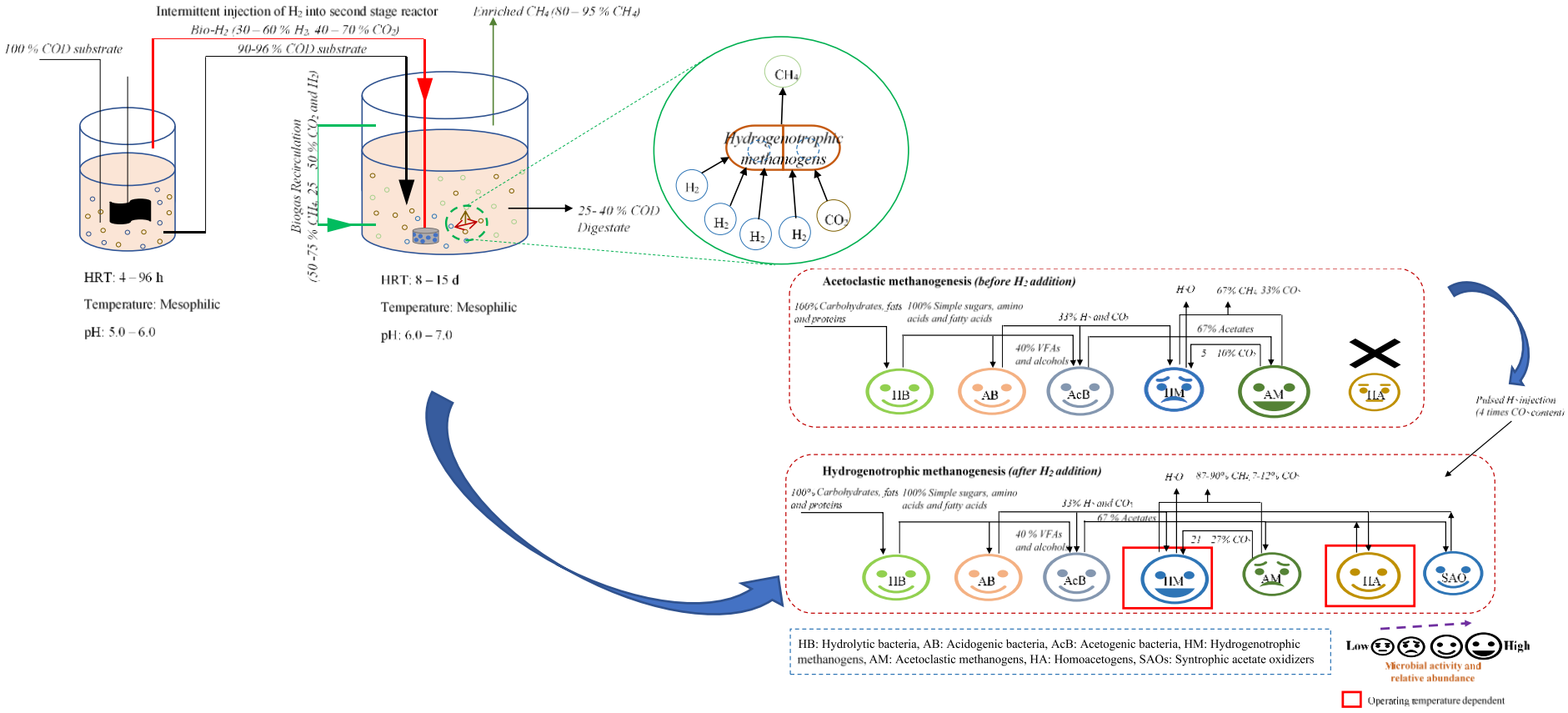
Word Count: 15255

Graphical Abstract

Innovative *in-situ* two-stage microbial methane enrichment



Possible microbial shift from acetoclastic to hydrogen assisted pathway

59 **Contents**

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List of Abbreviations

AB	–	Acidogenic bacteria
AcB	–	Acetogenic bacteria,
AD	–	Anaerobic digestion
AM	–	Acetoclastic methanogens
ASBR	–	Anaerobic sequential batch reactor
BESs	–	Bio-electrochemical systems
C/N ratio	–	Carbon-to-nitrogen ratio
CH_4	–	Methane
CO_2	–	Carbon dioxide
COD	–	Chemical oxygen demand
CSTR	–	Continuous stirred tank reactor
DF	–	Dark fermentation
FAA	–	Free acetic acid
FBR	–	Fed-batch reactor
FW	–	Food wastes
H_2	–	Hydrogen
H_2S	–	Hydrogen sulfide
HA	–	Homoacetogens
HB	–	Hydrolytic bacteria
HM	–	Hydrogenotrophic methanogens
HRT	–	Hydraulic retention time
MC	–	Moisture content
NH_3	–	Ammonia
OLR	–	Organic loading rate
SAOs	–	Syntrophic acetate oxidizers
sCOD	–	Soluble chemical oxygen demand
SFAOB	–	Syntrophic fatty-acids oxidizing bacteria
SMA	–	Specific methanogenic activity
TKN	–	Total Kjeldahl nitrogen

TOC	–	Total organic carbon
TS	–	Total solids
UASB	–	Upflow anaerobic sludge blanket reactor
VFAs	–	Volatile fatty acids
VS	–	Volatile solids

1. Introduction

Overexploitation of fossil fuels and accelerated energy demand substantially decreased their fuel abundance in the earth's natural reserves. The combustion of fossil fuels, in addition to unscientific solid wastes disposal, contributed 15 billion tonnes of carbon dioxide (CO_2) [1] and 30 to 70 million tonnes of methane (CH_4) [2] emissions annually, aiding global warming. Hence, major nations target adopting renewable energy production to cut off fossil fuels usage and greenhouse gas emissions, supporting the Kyoto protocol [3]. Biomass is an abundant renewable energy resource [4], capable of continuous energy production throughout the year [5], which is still in an expansion mode in terms of global energy production [6], also reducing the burden over conventional solid waste management practices.

Biomass to energy is accomplished either through thermochemical (for e.g., pyrolysis, gasification) [7,8] and biochemical methods (for e.g., anaerobic digestion (AD), composting) [6]. The multifaceted applicability of biofuel production and derived digestate as biofertilizer and carbon neutrality in nature prefers AD over other methods [1,9] from the late 1800s [10,11]. It is established through a series of four microbial pathways: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The biogas productivity during AD depends on the substrates used, microbial pathways, and environmental conditions [12]. Acetoclastic methanogenesis (AM) and hydrogenotrophic methanogenesis (HM) are the major methanogenic pathways contributing to CH_4 content in the biogas. In addition, another

methylotrophic pathway also exists negligibly consuming limitedly available methanol or methylamines to produce CH_4, CO_2 and water [1]. Biogas produced can be applied for cooking, lighting and thermal purposes and as a vehicular fuel. However, a prior enrichment of biogas is essential for the latter case since biogas is a mixture of various gases by volume: CH_4 (40 – 65%), CO_2 (35 – 55%) and other trace gas elements such as H_2S (0.1 – 3%), H_2, water vapour, siloxanes, etc. [13]. The enrichment of biogas improves its calorific value (> 90 % CH_4 content) and other fuel properties and confirmed the elimination of incombustible and corrosive gases [14].

Several biogas enrichment techniques based on physical and chemical methods have been successfully operated from laboratory to field-scale stages [15]. These techniques could be generally classified into two: CO_2 removal and CO_2 utilization techniques. The pressurized water scrubbing, chemical scrubbing using acids or bases, organic scrubbing, pressure swing adsorption, vacuum swing adsorption, membrane separation and cryogenic separation are categorized as CO_2 removal techniques [15–19]. In that sense, biological biogas upgradation based on CO_2 absorption through algal photosynthesis [20] also comes under this category. However, most of these techniques are either energy or cost-intensive, requiring advanced materials and equipment, high resource demand (water, chemical, etc.), and escalated capital investment [15,17]. More importantly, such techniques are preferable for large-scale plants considering the internal rate of return and system performance that depends entirely upon the biogas composition. Another limitation is the reduction in the volume of enriched CH_4 after the upgradation, which is a significant loss in a broader context. For instance, currently in India, biogas production is approx. 2.07 million m^3/year [21]. If this produced biogas is enriched through any of these upgradation techniques, 0.83 million m^3/year of CO_2 would be removed (considering 40% CO_2 content in biogas and 100% CO_2 removal). Other than that,

147 these techniques release the removed CO_2 openly into the atmosphere [16], while research on
148 the utilization of removed CO_2 for agricultural and industrial applications is underway.

149 Recently, microbial biogas enrichment has been on focus as a CO_2 utilization technique,
150 which converts CO_2 into CH_4 through specific hydrogen utilizing microbial species
151 (hydrogenotrophic methanogenesis and homoacetogens) [22,23]. Such enrichment methods
152 are technically feasible for all kinds of biogas plants irrespective of their working capacity if
153 they are successfully established, and the process parameters are rightly optimized [23]. From
154 the previous example, this technique has the potential to convert the entire CO_2 available into
155 CH_4 in the presence of H_2. Several reviews have been published previously on biological
156 biogas enrichment, including the fundamental mechanisms and comparison with other
157 technologies [12,16,24,25]. However, the possibilities of developing strategies for the startup
158 of a hydrogen-assisted pathway in existing systems for the microbial biogas up-gradation are
159 not yet reported and reviewed. This review discusses the prospects and challenges of
160 establishing microbial biogas enrichment through an innovative *in-situ* method for the co-
161 production of H_2 and CH_4 in an existing AD system. The review also explores the likely
162 syntrophic microbial activity pathways that might be achieved during the transformation from
163 AM to hydrogen assisted pathway.

164 **2. Anaerobic digestion: principles and governing factors**

165 Research and development over the AD process have improved rapidly following the energy
166 crisis in the 1970s [26,27]. Anaerobic digestion is usually carried out using a single substrate
167 (mono-digestion) and combinations of two or more substrates together, termed co-digestion.
168 Biochemical reactions taking place are crucial for the conventional AD process and the
169 positive advancement in microbial methane enrichment. Figure 1 shows the biochemical
170 process involved in the AD process. The hydrolysis process is the primary step (stage I) in

AD where complex structures (i.e., cellulose, lipids, carbohydrates, polysaccharides, proteins, and nucleic acids) experiences hydrolytic transformation using exo-enzymes secreted by facultative and obligatory anaerobic fermentative microbes [1]. The complex structures break down into monomers, simple sugars, saccharides, peptides, glycerol, amino and other higher fatty acids as in eq. (1).

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow n(C_6H_{12}O_6)\ \Delta G^{0'} = -215.67 \text{ to } -357.87 \text{ kJ} \ldots\ldots (1)$$

The initiation of the AD process depends upon the rate of hydrolysis as it directly influences the lag phase of microbes involved [1] and the chances for the formation of toxic by-products or a wide variety of non-desirable volatile fatty acids (VFAs) [28]. In the next acidogenesis step (stage II), the hydrolysed products get converted to VFAs such as acetates, butyrate, propionate, lactate, ethanol and other weak acids depending upon the partial pressure of H_2 and associated pH environment [29]. These by-products are further transformed into acetic acids, ammonia (NH_3), H_2 and CO_2 commonly through four major pathways (propionate, butyrate, lactate and ethanol) during the third stage, i.e., acetogenesis, a result of active acetogenic bacteria (stage IV) [29]. And also, acetate is produced along with H_2 in the acetogenesis stage through syntrophic fatty acids oxidizing bacteria (SFAOB) activity (stage III) [29]. Another pathway in acetogenesis is homoacetogenesis (HA), also known as the Wood-Ljundahl pathway, where H_2 and CO_2 are utilized to form acetates which further supports acetoclastic methanogenic (stage V) or syntrophic acetate oxidizing microbial activities (stage VI). Methanogenesis, the fourth stage, takes place after the acetogenesis stage (stage VII). In the process, the acetates, CO_2 and H_2 are converted into CH_4 by AM and HM. Among the microbial population contributing to AD, the growth rate of methanogens, in general, is slowest, which, in turn, demands an improvement in the hydrolysis rate and CH_4 content [29].

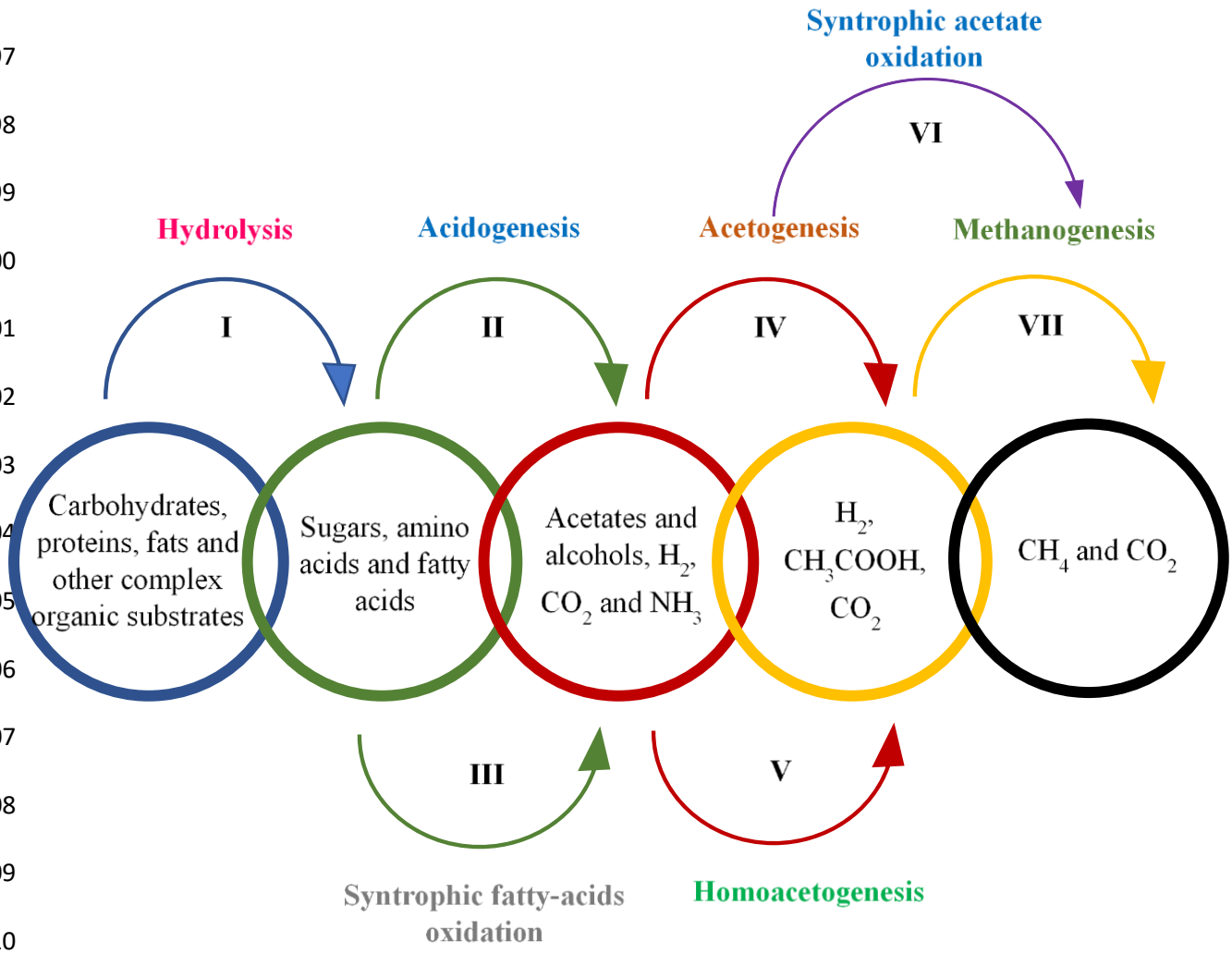

Fig. 1. The general biochemical process involved in anaerobic digestion.

On the other hand, the biochemical process involved in AD requires optimal operating conditions for proper microbial activity. The AD process occurs at a broader temperature spectrum, including mesophilic and thermophilic temperatures with an optimal temperature range between 20 - 65°C [30]. Meanwhile, the ideal pH range for hydrolysis is 6.0, acidogenesis 5.0 – 6.2, acetogenesis 6.0 – 7.0, and methanogenesis within 6.5 – 7.5 [31] with an optimum C/N ratio of 25 – 35 [32,33]. The pH variation in digesters relies heavily on alkalinity, VFAs, NH_3 concentration and the extent of available CO_2 inside the AD system [32]. Low pH around 4.0 favours VFAs production, while higher pH around 8.0 favours NH_3 production [34]. However, CH_4 production is inhibited when the VFAs and NH_3 accumulate

above 2000 – 6900 mg/L [35–38] and 80 – 1500 mg/L [36,39,40]. In contrast, another study reported that an NH_3 concentration less than 200 mg/L is beneficial for the AD process and stated that concentrations above the mentioned value inhibit both acetogenic and methanogenic microbial activity [41]. NH_3 concentration is directly related to the carbon-to-nitrogen (C/N) ratio of the substrate used [35], operating temperature and pH environment under anaerobic conditions [42,43]. Anaerobic digestion of substrates with lower C/N ratio values of 15.60 – 17.20 released NH_3 inhibiting the methanogenic activity and subsequently caused VFAs accumulation particularly, acetic acid [35]. However, at a desirable C/N ratio, NH_3 directly or in the form of ammonium (NH_4) enhanced the buffering capacity of the AD system [44] with a desirable alkalinity range within 1000 to 18000 mg/L [44,45]. Thermophilic microflora tolerance is expected to be active twice better than mesophilic microflora under NH_3 prone conditions [46]. However, another study witnessed that at thermophilic temperature (60°C), NH_3 adversely affected the biogas yield; however, it improved when the reactor temperature was lowered down to mesophilic temperature (37°C) [47]. Similarly, another study reported that NH_3 value surged notably with escalated pH at a controlled temperature [48]. The study observed that the NH_3 value hiked up to 10% at a pH value of 8.0 than 7.0 at a temperature of 35°C. Hence, the entire bioprocess in AD is relied upon several parameters and stabilized performance relied upon the control over these parameters during operation.

3. Improving methane content in the biogas through hydrogen assistance

3.1 Basics of hydrogenotrophic methanogenesis

In this pathway, HM reduces CO_2 for CH_4 production when H_2 or formate are provided as substrates, by indirect electron transfer from a cathode (e.g., zero-valent iron) and electricity termed as electro-methanogenesis or bio-electrochemical methane production [49] or by

direct electron transfer through syntrophic microbial activity [50,51]. Further, prior importance is given to interspecies/syntrophic microbial activity electron transfer and its effect on biogas composition during the introduction of H_2. In the general AD process, the HM pathway contributes to a maximum of 30 % of CH_4 content in the biogas composition with the lower levels of H_2 concentration available [52]. However, the dominance of AM and HM species depends upon the substrate used in AD [53]. When thermodynamic stability is considered, the HM pathway is more promising than the AM pathway [54]. The general stoichiometry of HM is autotrophic; it consumes CO_2 (one mole) as the sole carbon source and H_2 (four moles) as the electron donor to produce one mole of CH_4 (eq. 3). It is entirely different from the stoichiometry of the AM pathway, as shown in eq. (2). The detailed consumption pathway of hydrogenotrophic methanogenesis is elaborated in Lai et al. [21].

$$CH_3COOH \rightarrow CH_4 + CO_2 \ \Delta G^{0'} = -31.60 \text{ kJ} \ldots\ldots (2)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \ \Delta G^{0'} = -135.00 \text{ kJ} \ldots\ldots (3)$$

3.2 Understanding the biomethanation process

The application of HM based AD can be obtained through three approaches: (a) when H_2 is added directly into the reactor, *in-situ* method, (b) when H_2 is allowed to react with CO_2 in a separate reactor as a post-treatment, succeeding the anaerobic digester reactor, as *ex-situ* approach and (c) hybrid technology combining both *in-situ* and *ex-situ* approaches [55,56]. The *in-situ* method permits the transformation of existing biogas plants into the HM pathway to enhance CH_4 content in the biogas generated through direct H_2 addition. It has been reported that 65 – 100 % of CH_4 enrichment can be obtained through the *in-situ* approach under mesophilic conditions [57–60].

AD process through the *in-situ* approach directly exploits the differential solubility characteristics of CO_2 and CH_4 in the digestate liquor, makes it an economical way of biogas up-gradation. Meanwhile, in the *ex-situ* approach, the construction and operation of a second reactor significantly increase capital investments and hinders rapid application in the field. In addition, the post-treatment reactor should be equal or larger in size than the anaerobic reactor to accommodate the residence time of the H_2 gas, directly contributing to the capital investments [61]. The advantage of the *in-situ* technique over *ex-situ* and hybrid technologies is that it allowed utilization of existing infrastructures with slight modification for the upgradation of biogas [54] and reduced the expenses [22].

Table 1 shows the operational strategies and results obtained from different studies investigating *in-situ* methane enrichment. *In-situ* methane enrichment has been successfully investigated using the substrates, cattle manure [62], sludge and straw [57], cattle manure and whey [58,63], sewage sludge [59], swine manure [64], food waste [65], potato starch wastewater [66] and maize leaf [67]. Luo et al. [57] continuously fed H_2 into an anaerobic reactor treating cattle manure. A maximum hydrogen utilization efficiency of 79.72% and methane content of 65% was achieved at thermophilic conditions. The study concluded that the results could be improved if acidic waste streams are co-digested with cattle manure maintaining the pH around 7.0 and 8.0. The recommended pH range is essential for maximal hydrogen utilization through HM [62]. Thus, the same author later investigated the performance through co-digestion of cattle manure with whey, a known acidic substrate [58]. Maximum methane content of 75% was achieved with an H_2 utilization efficiency of 87.05% at the thermophilic range. A similar result was achieved in the case of swine manure with a methane content of 70% in the biogas composition at a mesophilic range, even though only an 8% increase in CH_4 content was achieved at a thermophilic temperature [64]. Much higher results were obtained using sludge and straw substrate and sewage sludge with 98.80 – 100 %

292 CH_4 content [57,59]. Overall, the studies recommended that successful development of the
293 *in-situ* approach relies on the seed sludge and substrates used, mixing rate, reactor
294 configuration, diffusers used, HRT, and OLR. The following section explains the technical
295 challenges and how various strategies improved the performance of the biomethanation
296 process.

297 **Table 1.** Reactor configurations, operating strategies and results of *in-situ* microbial methane enrichment studies

Reactor configuration	Temperature (°C)	Substrate used	Inoculum source	H_2 diffusion technique	Reactor volume (L)	pH	HRT (d)	Stirring speed (rpm)	H_2 utilization efficiency (%)	CH_4 production rate ($L/L_{reactor}.d$)	CH_4 enrichment (%)	References
CSTR	55	Cattle manure	Digestate manure	-	3.5	8.30	14	65	80	0.79	65	[62]
CSTR	38	Sludge and straw	Anaerobic sludge	-	2	7.90	20	1000	100	0.44	100	[57]
CSTR	55	Cattle manure and whey	Digestate sludge	Ceramic diffuser	0.6	7.89	15	150	87	0.89	75	[58]
CSTR	55		Digestate sludge	Column diffuser	0.6	7.74	15	150	81	0.76	53	
CSTR	55		Digestate sludge	Column diffuser	0.6	7.84	15	300	83	0.84	68	

CSTR	37	Primary and secondary sewage sludge	Digested sewage sludge	Hollow fiber membrane with coke oven	2	8.00	10	200	96	0.65	98.8	[59]
CSTR	35	Swine manure	Anaerobic sludge	-	11.2	7.59	25	228	18	0.78	70	[64]
CSTR	55		Anaerobic sludge	-	11.2	7.77	25	228	60	0.91	78	
CSTR	55	Cattle manure and whey	Digestate sludge	Hollow fiber membrane	0.6	8.30	15	150	22	0.90	96.1	[63]
FBR	37	Food waste	Anaerobic sludge	Ceramic diffusion	0.075	8.50	21	-	72	0.09	77.2	[65]

UASB	55	Potato starch wastewater	Anaerobic granules	-	3.5	8.40	20	200	94	1.15	82	[66]
FBR	52	Maize leaf	Thermophilic anaerobic digestate	-	0.12	7.00 - 8.00	24	100	100	0.13	89	[67]
Anaerobic filters	37	Maize silage hydrolysate	Process liquid digestate	Venturi nozzle	130	8.55	16	-	62	0.35	57	[68]
CSTR	37	-	Anaerobic digestate	-	3.5	8.60	90	350	99	0.145	94.57	[69]
CSTR	55	-	Anaerobic digestate	-	3.5	8.74	90	250	99	0.174	94.87	

298

4. Transformation of acetoclastic methanogenesis to hydrogen assisted pathways

4.1 Technical challenges occurred in hydrogen assisted pathways and likely solutions

Several studies monitored the start-up of HM-based AD under different operating strategies [57–59,64]. Still, most of these studies were limited to laboratory-scale due to the technical challenges linked with the process parameters. For instance, poor H_2 dissolution in the aqueous phase, which is crucial for HM reaction, directly affected the process performance [24] and the H_2 feeding above stoichiometric H_2: CO_2 ratio (4:1) accelerates the consumption and depletion of CO_2, directly affected the pH of the medium [62].

The H_2 gas-liquid mass transfer rate is typically expressed as (eq. (4)):

$$r_t = 22.40 \times k_L a(H_{2gTh} - H_{2l}) \ldots\ldots\ldots (4)$$

where r_t is the H_2 liquid mass transfer rate ($L/L_{reactor}$.d), 22.40 is the gas volume to mole ratio, k_La is the gas transfer coefficient per day, H_{2gTh} is the H_2 concentration in the gas phase (mol/L), H_{2l} is the H_2 dissolved in the liquid phase (mol/L).

Thus, from eq. (4) r_t can be enhanced by improving k_La [70]. In order to enhance r_t, several studies have investigated different strategies to improve its mass transfer rate inside the reactor. Continuous and stepped supply of H_2 through ceramic diffusers and hollow fiber membranes under continuous and intermittent stirring were monitored to improve the gas-liquid mass transfer during HM-based AD [57,59,62,64]. Increased H_2 concentrations induced by injections offer growth opportunities for HA with a higher H_2 affinity and HM with a low H_2 affinity, which would otherwise be outcompeted at the normal H_2 concentrations found in conventional anaerobic digesters [71]. Agneessens et al. [52] observed that about 61% of the injected H_2 was utilized for acetate production through the HA pathway. The study also emphasized that stepped feeding of H_2 gas is far more effective

than continuous supply achieving the complete conversion of CO_2 into CH_4 at HRT of 20 d and OLR of 0.77 g. VS/L. d. Through this strategy, the usual pH drop that is occurred due to high solubilization, partial pressure of H_2 and depletion of CO_2 could be eliminated. This is because one-time pulsed H_2 is not completely utilized for HM pathway alone even fed at controlled stoichiometric ratio, but also for cell synthesis and homoacetogenesis [57].

Similarly, the H_2 diffusion through the hollow fiber membrane is more effective than a column or ceramic diffuser [58,63]. A combination of venturi-based injection and external mixing was more effective as per a recently reported study[72]. However, the research on H_2 dissolution is still in the primary stages. As discussed earlier, the major constraints for establishing HM-based AD are the poor H_2 solubility and the extent to which H_2 injection may stimulate CH_4 production. In addition, it is strongly correlated to the mass transfer efficiency of the injected H_2.

Alfaro et al. [64] highlighted that elevated gas recirculation rates enhanced the H_2 gas-liquor mass transfer inside the reactor. At a high gas recirculation rate of 202 $L/L_{reactor}/d$, more prominent H_2 utilization was achieved than lower gas recirculation rates of 55 to 101 $L/L_{reactor}/d$. Agneessens et al. [52] optimized a sludge volume ratio of 2.5 % with a larger contact surface area with the headspace and intense stirring (~ 1000 rpm), being closest to a situation without gas-liquid mass transfer limitations. It was adversely affected when the sludge volume ratio was increased by more than 2.5 %. Zhu et al. [59] explained that the incorporation of intermittent stirring and lower H_2 feeding enhanced the CH_4 content in reactors irrespective of operating temperatures (mesophilic and thermophilic), overcoming gas-liquid mass transfer challenges. However, as the feeding mode changed from intermittent to higher H_2 feeding and intermittent to continuous stirring hiked the H_2 consumption more

345 significantly. Among the various reactor configurations, continuously stirred tank reactors
346 vastly improved the gas-liquid mass transfer limitations (Table 1).

347 The rapid consumption of CO_2 during H_2 injection leads to increased pH up to 8.3,
348 inactivating both the HM and AM [57]. Also, rapid CO_2 consumption and lack of its
349 availability inactivated the HM and HA [57]. These inhibitions were observed during the AD
350 of low organic content substrates(such as cattle manure) [54]. However, substrates with high
351 organic content, such as food wastes (FW) [65], sewage sludge [73] or co-digestion of
352 substrates together such as cattle manure and whey [58,63], could overcome this effect.
353 Alfaro et al. [64] observed that the elevated partial pressure of H_2 gas, pH rise (> 8.1), and
354 excess VFAs production did not inhibit the HM during biomethanation of sewage sludge.
355 Also, the performance in terms of VS removal was comparable to those without H_2 addition.
356 Okoro-Shekwaga et al. [60] investigated the HM-based mesophilic AD of FW. They
357 achieved a CH_4 enrichment of 77%. No inhibition on VFAs production and decomposition
358 occurred, which is directly associated with the AM activity when H_2 (gas mixture: 5% H_2,
359 95% N_2) was fed into a batch reactor. The increase in CH_4 content in the biogas composition
360 was about 12 %, which resulted in a 39 % reduction in CO_2. The study stated that the rapid
361 acidification potential characteristics of FW regulated the pH when CO_2 was depleted. Thus,
362 this present study further considers FW substrate as an example for *in-situ* biogas
363 upgradation, possible technicalities, parameters involved and likely solutions and is initiated
364 from the analysis of characteristics of FW and taking it further.

365 Proximate, elemental, and compositional characteristics of FW have been summarized in
366 Table 2. Since the composition of the FW is heavily dependent on the time, culture, habits,
367 region and seasons, the characteristics varied accordingly. AD of FW has been well studied
368 as a potential energy source due to its high organics and moisture content (MC). Generally,

FW contains MC of 48.80 – 94.36 % and TS around 5.64 – 51.20 %, with about 65.43 - 97.58 % of which are volatile solids (VS) [74–87]. FW is also composed of easily degradable carbohydrates (11.17 – 48.00 %), proteins (3.29 – 23.00 %) and lipids (2.33 – 23.00 %). Even though FW consists of macronutrients, however, FW is known for the lack of suitable C/N ratio (average: 17.50 ± 7.22) and trace elements [74–87]. With these characteristics, FW possesses a total biogas potential of 880.28 ± 12.90 L/kg. VS with a maximum CH_4 content of 55.19 ± 3.29 % and CO_2 content of 44.78 ± 3.29 % [74–87].

Table 2. Characteristics of food waste [74–87]

Parameters	Range	Average (SD)
pH	3.70 – 7.32	5.12 (1.31)
MC, %	48.80 – 94.36	78.25 (10.67)
TS, %	5.64 – 51.20	21.75 (10.67)
VS, %	3.69 – 28.02	17.94 (6.67)
VS/TS, %	65.43 – 97.58	90.32 (8.97)
sCOD, mg/L	2423 – 106600	69505.75 (46503.53)
COD, mg/L	103687 – 238500	114249.00 (119321.11)
TKN, mg/L	1.85 – 5.42	3.19 (1.56)
TOC, %	48.73 – 51.63	50.49 (1.54)
C/N ratio	4.80 – 55.00	20.38 (12.49)
Proteins, %	3.29 – 23.00	13.15 (13.94)
Lipids, %	2.33 – 23.00	12.67 (14.62)
Carbohydrates, %	11.17 – 48.00	29.59 (26.04)
Carbon, %	42.70 – 51.40	46.96 (4.35)
Hydrogen, %	6.10 – 9.10	7.60 (2.12)

Oxygen, %	38.90 – 46.20	42.55 (5.16)
Nitrogen, %	1.97 – 3.50	2.88 (0.80)
Sulphur, %	0.10 – 0.81	0.40 (0.37)
TBGP[a] (L/kg. VS)	871.16 – 889.40	880.28 (12.90)
TBMP[a] (L/kg. VS)	470.22 – 501.09	485.66 (21.83)
TBCP[a] (L/kg. VS)	369.90 – 418.98	394.44 (34.71)
CH_4 content[a] (%)	52.87 – 57.52	55.19 (3.29)
CO_2 content[a] (%)	42.46 – 47.11	44.78 (3.29)

TBGP- Theoretical biogas potential, TBMP- Theoretical biomethane potential, TBCP- Theoretical bio carbon dioxide potential, 'a' denotes the parameters calculated from the ultimate analysis values given in the Table using Buswell's equation.

The schematic representation of the interrelationship web of process parameters and its influence on stabilization during *in-situ* microbial methane enrichment when H_2 is injected into an AD reactor treating FW is shown in Figure 2. The acidification due to VFAs production during AD of FW prevents any pH rise under dominant HM activity. Hence, the addition of H_2 into the AD reactors treating FW has a high potential for enhanced CH_4 yield and biogas upgradation, supported by VFAs-induced pH buffering through the HM pathway. During the AD of FWs, apart from carbohydrates easily utilized for producing CH_4 and CO_2, high proteins and lipids available get transformed into VFAs, NH_3, CO_2, and H_2 [88–90], overcoming the depletion of CO_2 during HM activity. The release of VFAs leads to an initial reduction in pH and alkalinity that enhances the HM [65], while NH_3 and CO_2 help to retain a high amount of bicarbonate in the slurry mixture in the form of ammonium bicarbonate, thereby regaining the lost alkalinity as in eq. (5). It improves the buffering capacity inside the system.

$$NH_3 + CO_2 + H_2O = NH_4HCO_3 \ldots\ldots (5)$$

Meanwhile, the dissolved CO_2 is again utilized by HM to produce CH_4 in the presence of H_2 (Eq. 3), buffering the low pH-induced by high VFAs production [65]. On the other hand, NH_3 can also react with VFAs (C_xH_yCOOH), especially at a higher organic loading rate (OLR), inducing buffering capacity according to the eq. (6) [91].

$$C_xH_yCOOH + NH_3 + H_2O \rightarrow C_xH_yCOO^- + NH_4^+ + H_2O \ldots\ldots (6)$$

Hence, the pH environment during the AD is controlled by an overall set of reactions inclusive of bicarbonate formation, NH_3 accumulation, and VFAs production and degradation.

Another inhibitory factor is the presence of free acetic acid (FAA), a predominant contributor to the VFAs component that affects the AM [92], a result of higher OLR [93]. It was reported that the specific methanogenic activity (SMA) of a mixed culture of AM and HM inhibited up to 50 % at 0.31 g/L and 90 % at 2.36 g/L of free acetic acid concentration [92]. Furthermore, the study found that specific methanogenic activity of the mixed culture was strongly correlated with the free acetic acid according to the eq. (7):

$$SMA\ (gCH_4/gVSS.d) = \frac{(0.86 \times 0.31)}{(0.31 + FAA(mg/L))} \ldots\ldots (7)$$

where, the value 0.86 is the reaction constant, K, and the value 0.31 is the FAA with SMA equal to 0.5 g/L ($FAA_{50\%}$).

These conditions are generally observed under AM and HM dominant environments. Thus, the concern is on the probable inhibition of AM due to potential inhibitory effects of VFAs particularly, acetates and NH_3, at higher concentrations and have no control over these parameters. It results in reduced CH_4 yield and eventual digester breakdown in the long run [94,95]. On the other hand, increased OLR up to 2 g VS/L. d along with acetic acid

accumulation and high H_2 partial pressure propitiated the HA species [96,97] and syntrophic acetate oxidizing bacteria (SAOB) activities [29]. Hence, the development of *in-situ* microbial methane enrichment has relied on a syntrophic microbial activity through HA-AM and SAO-HM pathway than being specific to normal AM and HM activity. Under a high H_2 partial pressure environment or high OLR, HA utilizes 4 moles of H_2 and 2 moles of CO_2 producing 1 mole of acetate (eq.8) or direct utilization of monomers to produce acetates (eq.9) and subsequently, either SAOB reverses the process (eq.10), or under favorable conditions, AM utilizes the produced acetates (eq.2). The HA-SAO pathway can act as temporary energy storage for H_2 under unfavorable AD conditions that could be converted to CH_4 when the favorable conditions initiate at H_2: CO_2 ratio of 4:1 utilizing the accumulated acetates [57]. The thermodynamic stability of the mentioned pathways depends upon the operating temperature; thermophilic (55℃) is most suitable than mesophilic (25℃) [98,99].

$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$…….(8) $\Delta G^{0'}$= -104.60 kJ (Autotrophic homoacetogens)

$C_6H_{12}O_6 \rightarrow 3CH_3COO^- + 3H^+$……(9) $\Delta G^{0'}$= +310.90 kJ (Heterotrophic homoacetogens)

$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2$……(10) $\Delta G^{0'}$= +104.60 kJ (Syntrophic acetate oxidizers)

Although the Wood-Ljungdahl pathway is not ideal, the phenomenon is expected to outcompete HM since HA are known for their higher specific H_2 consumption [100]. The study saw that at H_2: CO_2 ratio of 4:1 and an H_2 partial pressure of 0.96 bar, HM and HA consumed H_2 at a proportion of 60 and 40%, respectively, thus increased the CH_4 production via the Wood-Ljungdahl pathway [100]. However, a long-term operation and repeated pulsed

H_2 injection stimulated HM over HA activity even at a high OLR of 2 g. VS/L. d [96]. The study cited that the advantage of thermodynamic stability that HM consists over HA contributed to their proliferation. The hydrogen consumption pathway can also be linked with the operating temperature as Zhu et al. [59] observed that at thermophilic temperature (55°C), HA dominated over HM activity with much shift in microbial consortia than at mesophilic temperature (35°C).

Similarly, during the process start-up, promotes faster degradation of propionate through the enhanced activity of the H_2-consuming bacteria, thereby reducing likely propionate-induced inhibitions [101]. Another study reported accelerated degradation of both propionate and acetate within 10 days when a higher concentration of H_2 was injected [67], which is supported by other studies [62,66]. Likewise, prolonged operation (200 d) of an unstable AD reactor treating FW at an OLR of 1.6 g. VS/L. d reversed propionate accumulation through HM pathway under high H_2 partial pressure when feeding strategy was changed from continuous to pulsed [53]. However, the optimization of inhibitory parameters still lacking proper investigation and could be adjusted only through intensive evaluation by long-term advanced laboratory and pilot-scale studies.

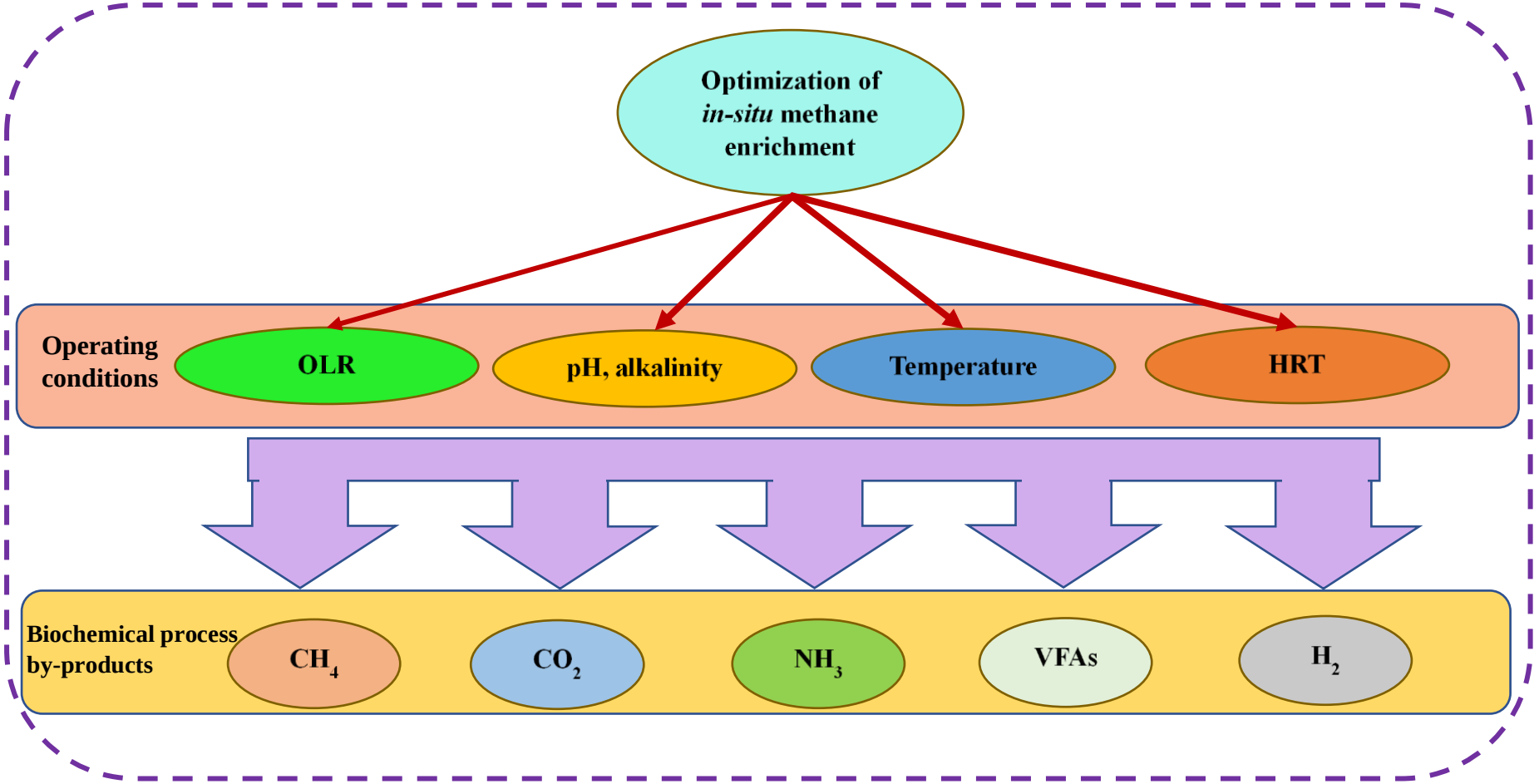

Fig. 2. Inter-relation web of process parameters and its influence on stability during anaerobic digestion of FWs.

Different innovative strategies have been reported to optimize the process parameters in anaerobic digestion for long-term operation. The NH_3 stripping [102–104], the addition of trace elements [105–107], amendment of carrier materials [108–113], leachate recirculation [114] and intermittent micro-aeration [115,116] have been investigated to improve the process stability and CH_4 yield from FW. NH_3 stripping through bubbling of biogas directly into the slurry resulted in maximum NH_3 removal of 4.5 - 10.4% per day at a temperature range of 35 - 70 ℃ and found that even a small amount of NH_3 removed is sufficient to allow the microbial consortia to operate more effectively [102]. Trace element addition yielded 465 mL CH_4/ g. VS_{added} from FW with no hints of VFAs at an OLR of 1.0 – 5.0 g VS/ L. d [105]. The incorporation of carrier materials such as cermasite, filter media, the combination of vermiculite shells and granular perlite, and biochar improved the CH_4 content of up to 95% [108–110] and sequestrated 51 - 61 % of CO_2 in the case of biochar [108]. The availability of cations and nutrients, including calcium, magnesium, sodium, and potassium in trace elements, stimulated the microbial synergy, and carrier materials stabilized the digestion of FWs. Sequestrated CO_2 and enhanced specific surface area in biochar amended reactors may improve the contact time of H_2 in the slurry, and the chances of the microbes to survive even under unsuitable environmental conditions [117]. Biochar can also potentially act as a pH neutralizer, and a redox-active mediator stimulating the direct transfer of electrons between syntrophic microorganisms and inhibits H_2-based syntrophic pathways simultaneously [118], improving the activity of both AM and HM [109,110].

The recirculation of leachate at a dilution ratio of up to 0.50 stimulated methanogenic activity and caused enhanced biogas generation during AD of FW [114]. Recirculation of this digestate effluent also reduces the freshwater requirement in the system. The performance was negatively affected when the dilution ratio increased above 0.50. In another study,

intermittent micro-aeration was effectively used as a strategy to enhance the hydrolysis rate, CH_4 yield, VFAs production, and consumption and H_2S removal at higher OLR and without pH buffering [115,116]. High precise oxygen-dosing systems are required in this strategy, which may not be economically feasible for small-scale digesters. Co-digestion of acidic substrates or increased supply of H_2 gas is also considered as an excellent option to overcome the challenges faced (i.e., rise in pH and VFA accumulation) during the biomethanation using HM [62].

Table 3. Technical challenges and likely solutions for the successful development of *in-situ* microbial methane enrichment

Technical challenges	Reasons	Likely solutions	References
Failure treating low organic substrates	Faster CO_2 depletion	Co-digestion with suitable substrate	[63,64]
High H_2 partial pressure	AM inhibition	Pulsed H_2 and substrate feeding	[53,57,62]
Low H_2 gas-liquid mass transfer	Limited hydrogen assisted pathway reaction	Biogas recirculation, low sludge volume ratio, HFM diffusion and pulsed H_2 feeding	[57,63,102]
Rapid consumption of CO_2 and subsequent rise in pH and low buffering capacity	Inhibition of AM and hydrogen assisted pathways	Use of high organics substrates or co-digestion followed by	[63–65,73]

		likely VFAs and NH_3 production	
Requirement of high OLR, acetate accumulation and propionate degradation	AD process hindering	Propitiation of HA-AM or SAO-HM pathway	[64,100]
Selection or controlling the H_2 consuming (HA or HM) pathways	For enhanced performance	Control over operating temperature (mesophilic or thermophilic) and long-term reactor acclimatization	[22,64]
Long term reactor acclimatization and stabilization	Process stabilization and microbial acclimatization	Biochar amendment and biogas recirculation	[73,102,108]

504

505 *4.2 Microbial interaction during transformation from acetoclastic to hydrogenotrophic*
506 *methanogenesis*

507 In general, the anaerobic digestate in the AD reactor comprises 93 – 98 % of bacteria and
508 0.30 – 7.10% archaea [67,119,120]. During hydrolysis, microbial species such as *Clostridia*,
509 *Bacteriodetes*, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* actively help solubilize
510 complex organic structures into monomers [121,122]. Additionally, acetogenic and

511 syntrophic bacteria of *Firmicutes* and *Proteobacteria* sp. degrades these organics into VFAs
512 and other weak acids [122]. Some of the *Actinobacteria* sp. contribute to VFAs and
513 propionate production along with hydrolysis [123]. *Lactobacillus, Clostridium, Pediococcus,*
514 and *Streptococcus* play a vital role in hydrolysis and acidogenesis [121]. These species are
515 the major contributors to H_2 production in the AD process [124]. *Acetogenium* sp. and
516 *Syntrophococcus* sp. are the potential acetogens supporting the acetogenesis stage and
517 homoacetogens such as *Clostridium aceticum* sp. and *Acetobacterium woodii* sp. taking the
518 Wood-Ljungdahl pathway [125]. About 54 – 72 % relative abundance of microbial species
519 present in the anaerobic digester supports the stages of hydrolysis, acidogenesis and
520 acetogenesis [53,67]. In conventional AD, the methanogenic microbial population consists of
521 *Methanomicrobiales*, *Methanobacteriales,* and *Methanosarcinales,* which are very active
522 reported up to 95 % relative abundance at the genus level methanogenic species with a co-
523 existence of HM communities such as *Methanobrevibacter*, *Methanobacterium,* and
524 *Methanosphaera* [57]. Meanwhile, the high availability of organics initiates a significant shift
525 from AM to HA due to available acetate accumulation and outcompete HM inside the reactor
526 [73]. Likewise, similar conditions inhibit AM species *Methanosaeta*, which is vulnerable at
527 elevated acetate concentrations, while *Methansarcina* with a higher growth rate at similar
528 conditions is expected to dominate [126]. In contrast, under the high availability of organics,
529 Li et al. [112] observed an increased abundance of *Tenericutes*- affiliated bacteria, which are
530 considered as facultative anaerobes and produce organic acids utilized by AM. Hence under
531 an optimized AM dominating environment, the existence of HA is negligible, with only 2 – 5
532 % of H_2 is consumed by HA [127].

533 The introduction of H_2 into the reactor does not cause a significant change in the rate of the
534 first three stages in AD (hydrolysis, acidogenesis and acetogenesis) even though there can be
535 changes regarding prominent bacterial species among the population and the bacterial

metabolism [128]. Zhu et al. [59] observed that the addition of H_2 into the reactors enhanced the relative abundance of *Firmicutes* over *Bacteriodetes* with its ability to strive under high H_2 partial pressure. Also, the *Treponema_2* and *Terrisporobacter* (*Clostridium sp.*) known homoacetogens were found in abundance [59,64]. The addition of H_2 also caused a shift towards HM species of *Methanobacterium* and *Methanobacteriales_OTU_16* with a more diverse microbial consortium [57]. Besides, the *Methanomicrobium* genera increased substantially after H_2 addition at an H_2: CO_2 ratio above 4:1 [57,67]. Within a short interval of time after H_2 injection, Kakuk et al. [126] observed that the activity of the *Methanoculleus* genus related to HM substantially increased. Agneessens et al. [52] also observed that the relative abundance of genera *Methanosarcinales* (capable of acting as AM and HM) reduced gradually from 10.20 % to 7.8 % at the end of the experiment in H_2 injected reactors, instilling a gradual shift towards HM species. Under thermophilic conditions, the unusual syntrophic activity of *Desulfovibrio* sp. that produces acetate, H_2, and CO_2 under limited sulfate conditions and HM increased and reduced the microbial population diversity [52].

From the externally added H_2, around 40% of H_2 are reported to be consumed by the HA, significantly contributing to the Wood-Ljungdahl pathway [100], since the half-velocity constant of HA for H_2 is ten times higher than HM [130]. However, long-term acclimatization of the digester may entertain AM due to the readily available acetates produced by HA, enhancing CH_4 production and stabilizing the HM pathway over time [29]. Furthermore, it leads to the same stoichiometric biogas upgrading equivalent to the stoichiometric HM reaction [59]. But, an increase in pH favors the HA [131] and decreases the AM activity [132]. Additionally, partial H_2 pressures at lower values of 6 kPa further hinder acetate consumption by AM species [133,134] and contribute to acetate build-up during H_2 injection [67].

Temperature plays a vital role in the initiation of these microbial activities. High bacterial and archaeal population diversity was found at mesophilic temperature (30 – 40 °C) than thermophilic temperature (50 – 60 °C) [135,136]. Zhu et al. [59] compared the microbial population shift observed in thermophilic and mesophilic hydrogen-assisted *in-situ* microbial methane enrichment reactors. Table 4 shows the major microbes identified after H_2 was injected into the reactor. The study classified microbes into five according to the biochemical processes they involved with: (a) hydrolysis, acidogenesis and acetogenesis, (b) SFAOB, (c) SAOB, (d) HA, and (e) methanogens. The study observed that as the operating temperature is increased from mesophilic to thermophilic, the hydrolysis and acidogenesis rate accelerate, contributing to increased VFA concentration. Hence, the relative abundance of *Methanosaeta* sp. reduced substantially from 12.60 % at 35℃ and 0.06% at 55℃. This resulted in flourish of SAOB coupled with HM in thermophilic reactors. In contrast, hydrogen pathway in mesophilic reactor promoted Wood-Ljungdahl pathway over SAO-HM pathway assisting AM pathway. However, the methane yield from both the reactors were comparable (Table 1). Figure 3 shows the possible microbial pathway in a conventional AD reactor and H_2 assisted AD reactor conceptualized and modified from the literature. Thus, it could be concluded that a syntrophic activity between HM, AM, SAO, and HA must be co-existing inside a reactor to achieve higher CH_4 content in the biogas composition through hydrogen assisted pathways. However, more research is required for the optimization of the system, since the microbial population are highly influenced with the seed sludge used and other operating parameters.

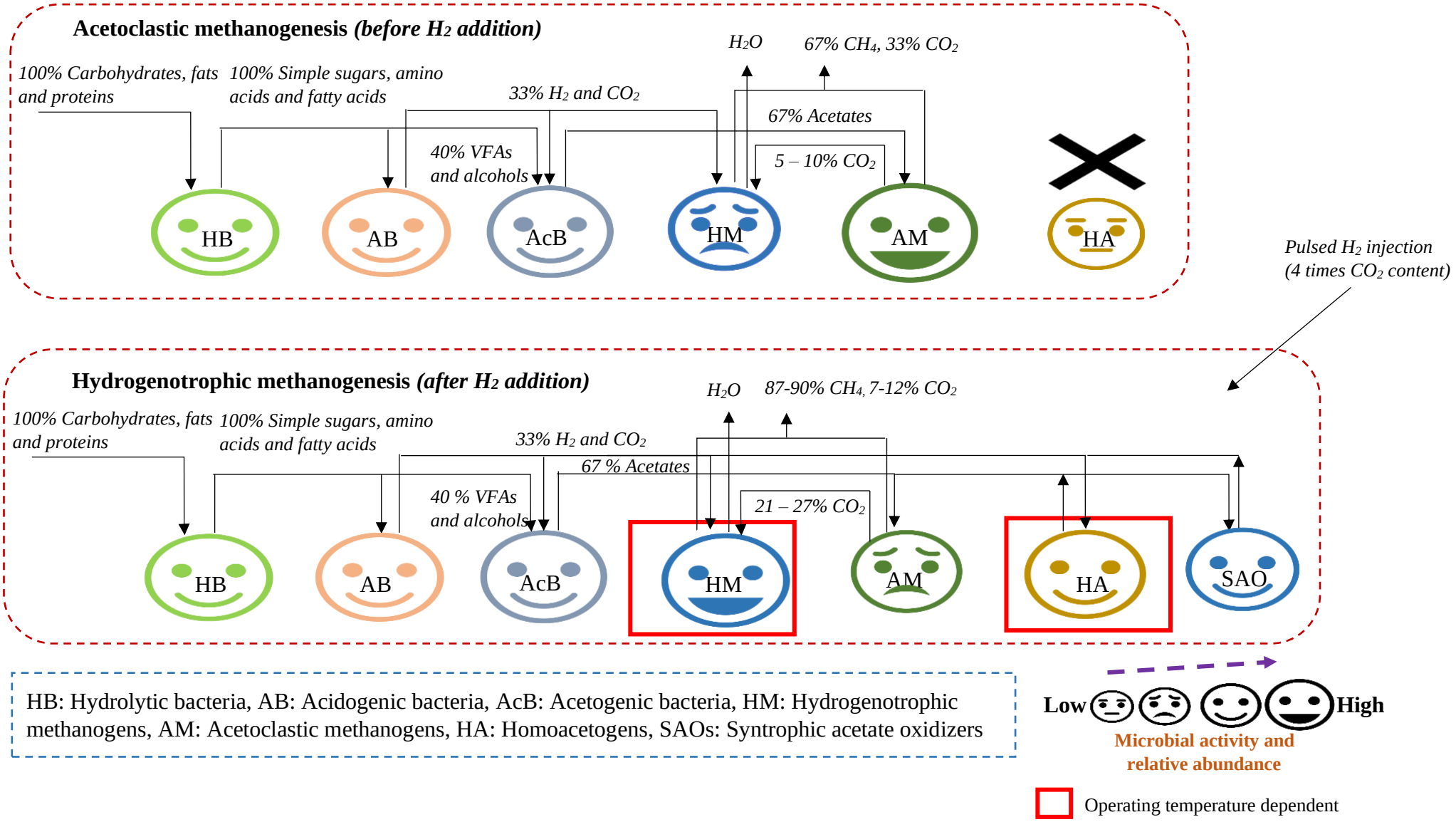

Fig. 3. The microbial pathway during acetoclastic methanogenesis and after transformation to hydrogenotrophic methanogenesis pathway (quantitative values and relative abundance of the microbial population were taken from [29,52,67]).

596 **Table 4.** Comparison of dominant microbial population in mesophilic and thermophilic *in-situ*
597 microbial methane enrichment reactors (Relative abundance is greater than 1) (taken from [64]

Biochemical process	Thermophilic reactor	Mesophilic reactor
Hydrolysis, Acidogenesis and Acetogenesis	*Psychobacter, Ruminofilibacter, Ruminiclostridium_1, Norank_o_MBA03, Clostridium_sensu_stricto_1, norank_f_Family_XI*	*Clostridium_sensu_stricto_1, vadinBC27_wastewatersludge_group, Treponema_2, Streptococcus, Marinilabiaceae, Christenellaceae R-7 group*
SFAOB	*Syntrophomonadacaea, Synergistaceae*	*Syntrophomonas*
SAOB	*Gelria*	-
HA	-	*Terrisporobacter, Treponema_2*
AM	*Methanosarcina*	*Methanosaeta*
HM	*Methanoculleus, Methanobrevibacter, Methanobacterium*	-

598

599 **5. Preferential ways for H_2 production aiding hydrogen assisted microbial pathways**

600 From the discussions, it is clear that the availability of H_2 is the primary factor required for
601 the transformation from AM to HM-based AD. Thus, the economical, efficient, and
602 sustainable production of H_2 is necessary to keep the system more practical for the
603 application. Various studies focussed upon the H_2 production using water electrolysis [137],

gasification [138], bio-electrochemical systems (BESs) [49] and microbial pathways of fermentation [139,140]. In water electrolysis, H_2 is produced by splitting water into O_2 and H_2 [137], which can also be a potential storage mechanism of surplus electricity in the form of H_2 [62]. Gasification converts carbonaceous organic materials into a mixture of gases mainly composed of carbon monoxide, H_2, and CO_2 completely with slight traces of ash [138].

Meanwhile, BESs integrated water electrolysis inside the AD reactor for cathodic electron transfer propitiating the HM activity. Water electrolysis can be an economical option considering electricity used is produced from renewable energy sources or surplus electrical energy [23,25]; however, the requirement of a continuous supply of electricity and water is a concern. Moreover, the need for electricity is also a concern for BESs as well. Also, a portion of the energy produced from the AD system should be stored or fed as input energy for the water electrolysis and BESs. Meanwhile, gasification needs much controlled operating conditions and leads to the complete utilization of substrates.

Hence, a non-powered biological production of H_2 possesses a significant upper hand over other techniques such as water electrolysis and gasification. Cyanobacteria and algae may produce H_2 through bio-photolysis of water [141] or by photosynthetic and chemosynthetic fermentative bacteria. To initiate these techniques through a continuous supply of photo-energy (natural or artificial) is itself a challenge. Anaerobic fermentative bacteria produce H_2 without photo-energy. Thus, the cost of H_2 production is lower than the photosynthetic process (about 340 times less), only requiring simple reactor configuration and continuous production and yield per unit of the reactor [142,143]. Another added advantage of this anaerobic process, termed dark fermentation (DF), is that it is already an intermediate process within the AD process. Also, it is well known that carbohydrates are the primary source of H_2

during fermentative processes. Therefore, any organic waste rich in carbohydrates can be considered the potential source of H_2 [144]. An intensive literature review of possible H_2 production through the DF pathway is earlier reported [145,146]. The production of H_2 through the DF also results in acetic and butyric acids followed by ethanol and acetic acids as by-products [140,147]. The two stoichiometry pathways of H_2 production from the available simple sugars are given in eq. (11) and (12).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 4H_2 + 2CO_2 \quad \Delta G^{0'} = -206.00 \text{ kJ} \ldots\ldots\ldots (11)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_4H_8O_2 + 2H_2 + 2CO_2 \quad \Delta G^{0'} = -254.00 \text{ kJ} \ldots\ldots\ldots (12)$$

From eq. (11), the maximum H_2 yield is about 4 moles from one mole of hexose or 33% H_2 recovered from the substrate when acetic acid is the only by-product. On the other hand, if butyric acid is the only fermentation by-product, the maximum H_2 yield is only 2 moles from one mole of hexose, or 17% H_2 recovered from the substrate (eq. 12) as experienced during the co-digestion of organic fraction of MSW and sewage sludge [139]. Similar to the CH_4 yield, the actual H_2 yield is always lower than the theoretical value since the substrate is often converted to other metabolic products and biomass [148] and is usually observed below 20 % [149]. In contrast, [111] reported lower H_2 yield in the range of 5 – 10 % (as in eq. 8) and VFAs production. It would mean another pathway, as shown in eq. (13) where no production of H_2 takes place, as the substrate is consumed for lactic acid production. Thus, carbon sources completely convert into lactic acid instead of H_2 under lower pH (< 4.5) [150].

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 \quad \Delta G^{0'} = -225.4 \text{ kJ} \ldots\ldots\ldots (13)$$

Inhibition of H_2 consuming bacteria such as HM, HA, lactic acid bacteria, propionate producing bacteria and sulfate reducers is one of the main steps for initiating DF-based H_2 production when using inoculum consisting of mixed microbial communities. Thermal [151–

153] and chemical [111,139,154] pre-treatments were applied to enrich H_2 producing bacteria and inhibit remaining microbial competitors successfully. Table 5 shows the summarization of the results obtained from the DF of food wastes.

654 **Table 5.** Summarization of dark fermentation studies for bio-H_2 production

Substrate used	Optimal operating conditions	Maximum bio-H_2 yield	References
Food waste	Reactor volume: 1.20 L Inoculum used: Chemically pretreated sewage sludge Substrate to inoculum ratio: 2.40 VS basis Stirring: 120 rpm Addition of bottom ash: 1 g/L Temperature: 37℃	169 mL/g. VS_{added}	[155]
Food waste	Reactor volume: 5.00 L Inoculum used: Untreated activated sludge Substrate to inoculum ratio: 0.14 VS basis Stirring: 150 rpm Temperature: 39℃ pH: 6.50	88.8 L H_2/kg.VS	[156]

Crude cheese whey and fruit vegetable waste	Reactor volume: 1.80 L Inoculum used: Untreated activated sludge C/N ratio: 21 Temperature: 37℃ pH: 5.50	449.82 mL H_2/gCOD	[157]
Mixed food waste	Reactor volume: 500 mL Inoculum used: Untreated anaerobic sludge Food to microorganism ratio: 7 to 10	57 mL H_2/g.VS	[158]

655

6. Co-production of H_2 and CH_4: the way to move forward?

Investigations were reported over the simultaneous production of H_2 and CH_4 in two-stage reactors from the past few decades: hydrolysis to acetogenesis in the first stage and methanogenesis in the second stage [124,149,151–153]. The essential mechanisms involved in the AD bioprocess primarily depend upon the acidogenesis and methanogenesis stages require varied nutritional requirements, pH environment and growth kinetics [159]. Hence, two separate digesters for both processes can uplift the process efficiency over a single-stage AD process [160]. The improved process efficiency is also because of no direct influence of VFAs or NH_3 disrupting the microbial activity since the processes occur in two separate reactors [161,162]. The by-products from the first stage reactor comprised of VFAs prominently acetates, H_2 and CO_2 [111,124,151–153]. Acetate is a crucial intermediatory product during acidogenesis, and acetogenesis contributing to a significant part of the CH_4 produced [29] later in the second stage reactor. In two-stage AD, pH is a determinant factor in the first stage, deciding the production of desirable by-products through selective microbial metabolic pathways [163]. An optimal pH 5.5 in the first stage reactor supports the production of acetic acid followed by butyric acid and propionic acid through the pathways, which is essential for enhanced H_2 production, as discussed earlier in section 4.3. Maximizing the acetate production during acidogenesis requires either pH adjustment, OLR regulation and subsequent control over H_2 partial pressure inside the reactor [29].

Table 6 summarizes the operating strategies used and results obtained for the simultaneous production of H_2 and CH_4 from different substrates. During AD of FW, only 4% of the total COD is utilized for H_2 production in the first reactor, while about 55% of the COD is converted into CH_4 in the second reactor. It also depended upon the operating temperature [153] and was 16% less in the first stage and 25% less in the second stage during the AD of

sweet sorghum, as reported by [149]. Other studies summarized in Table 3 also reported similar values and observed CH_4 to H_2 production at a ratio between 1.33 to 41.95 for two-stage AD [151–153]. This ratio was significantly influenced by the operating strategies, environmental conditions, and reactor configurations. The volume ratio between the first and second stages is an essential factor affecting the H_2 and CH_4 yield. In comparison to all the studies, a maximal H_2 and CH_4 production of 292.70 and 391.60 mL/g VS was achieved in two-stage CSTR at an OLR of 48 g COD/L d treating FW when the working volume of both the reactors was kept the same [124]. The concept of two-stage AD also lowered the H_2S content in the biogas through limiting the sulphate reducing bacteria [164] by nitrate addition at a nitrate to sulphide ratio of 16: 1 [165], and also aids in satisfactory pathogens removal [166].

691 **Table 6.** Operation strategies and results from co-production of bio-H_2 and CH_4 from two-stage anaerobic digestion

Reactor Type (volume, in L)	Substrate used	Inoculum Used	Operating conditions	Bio-H_2 produced in first stage	CH_4 produced in second stage	References
CSTR (0.07 L x 2)	Sugarcane syrup	Thermally pretreated UASB granules Second stage: Non-pretreated UASB granules	HRT: 2.5 – 2.75 d (1^{st} stage), 45 d (2^{nd} stage) Temperature: 30 ℃ pH: 6.5 (1^{st} stage), 7.0 (2^{nd} stage)	88 L/kg. VS	271 L/kg.VS	[149]
FBR (0.417 L x 2)	Ozone pretreated glycerol trioleate and FW	First stage: Thermally pretreated activated sludge Second stage: acclimatized activated sludge	HRT: 3 d (1^{st} stage), 39 d (2^{nd} stage) Temperature: 35 ℃ pH: 6.0	22.55 mL/g.VS	946 mL/g.VS	[151]

CSTR (4.5 L x 2)	FW	First stage: Thermophilic (55 °C) anaerobic sludge Second stage: Mesophilic (35 °C) sludge	HRT: 3 d (1st stage), 39 d (2nd stage) Temperature: 55 °C (1st stage), 35 °C (2nd stage) pH: 6.0	104.50 L/ g VS	526 L/g VS	[153]
CSTR (first stage, 5.0 L) UASB (3.5 L) and ASBR (3.0 L) (second stage)	Thermo-chemically (4.80 % HCl, 93 °C) pretreated *Laminaria japonica*	First stage: Thermally pretreated (90 °C) anaerobic sludge Second stage: anaerobic sludge	HRT: 2.7 d (1st stage), 2-12 d (2nd stage) Temperature: 35 °C pH: 5.5 to 8.0	115.20 mL H_2/g dcw	330.20 (UASB), 226.50 (ASBR) mL/ g COD	[152]
CSTR (first stage, 1.34 L), CSTR	Diluted organic market waste	First stage: Chemically	HRT: 1.1 – 1.5 d (1st stage), 11 – 15 d (2nd stage) Temperature: 35 °C	74 mL/ g VS	179 mL/g VS	[154]

(second stage, 13.4 L)		pretreated (2 M HCl) cow manure Second stage: Cow manure	pH: 5.5 (1^{st} stage), 7.0 (2^{nd} stage)			
CSTR (4 L x 2)	FW	First stage: Thermally pretreated (100 °C) anaerobic sludge Second stage: anaerobic sludge	HRT: 12 h (1^{st} stage), 24 h (2^{nd} stage) Temperature: Mesophilic pH: 6.0 (1^{st} stage), 7.0 (2^{nd} stage)	292.70 mL/ g VS	391.60 mL/g VS	[124]

692

7. Possible concept for successful development of hydrogenotrophic methanogenic based reactor

A possible concept of two-stage H_2 and CH_4 production and mass balance when FW is used as the substrate is represented in Figure 4. A two-stage AD could be designed to separate the involved microbiomes into two bioreactors, as discussed earlier in section 6. The acidification process inside the first stage reactor is rapid. Hence, it requires a lower HRT (4 – 96 h) [149,163] with an optimal pH range of 5.0 to 6.0 [163]. Reduced HRT in first stage reactor have several advantages: enhances the acetogenic bacterial activity [167], scales down the volume of first stage reactor by 2.25 to 10 times smaller than the second stage reactor [154,164,168], eliminates any chances of competition between H_2 producing acetogens and HM in the first stage reactor [154]. Integrating the first stage reactor with the second stage reactor is also possible, beneficial for reducing the requirements of additional mechanical attachments or machines if the first stage is externally operated. A similar concept of integrating two stages inside a reactor was designed and successfully operated to hydrolyse fruit and vegetable wastes [169]. Since bio-H_2 production from one-time feeding of the substrate is lower than equivalent CH_4 production from the same amount of feed in the second stage reactor, an establishment of more than one first stage reactor could also be an option to consider for the simultaneous operation to achieve the hydrogenotrophic stoichiometry of 4: 1 (H_2: CO_2). After initial digestion (hydrolysis, acidogenesis, and acetogenesis), the substrate ultimately breaks down into acetates and results in continuous H_2 and CO_2 production. Following each feed, the digested substrate from the first stage reactor is transferred to the second stage reactor, where methanogenesis reactions occur. The good methanogenesis in the second stage results in significant production of CH_4 and CO_2, which is relied upon the HRT of the first stage reactor and acetate input level into the second stage reactor. Hence, the common gas collection section contains biogas containing H_2 and CO_2

718 from the first stage, CH_4 and CO_2 from the second stage. It would also eliminate any separate
719 storage system for H_2 and the safety concerns associated with it. To improve the hydrogen-
720 assisted pathways, the biogas recirculation arrangement facilitates the consumption of H_2 and
721 CO_2 at the required ratio. The biogas recirculation system improves the biogas yield (55%),
722 CH_4 content (26%), and COD removal (86 – 99%) [10]. Intermittent feeding of H_2- rich
723 biogas into the methanogenic reactor slurry could proliferate the HM [57]. From the
724 discussion made, it is understood that the biogas recirculation enhances the co-existence of
725 AM, HA, SAOs and HM [73], and helps in stripping off the accumulated NH_3 [102],
726 eliminates the requirement of a separate storage system for H_2, and could instil an
727 intermittent motion or suspension inside slurry serving homogenous mixing. Recirculating
728 the mixture of biogas could also eliminate the possible pH variation in the slurry due to CO_2
729 consumption and excess H_2 injection [73]. The utilization of biochar in the second stage
730 reactor is also an option that could be considered with its positive characteristics as
731 previously discussed.

732 During anaerobic digestion, stoichiometric molar production of CH_4 of up to 67 % through
733 AM followed by CO_2 and H_2 of 33% contributing directly to hydrogen assisted pathways
734 [29]. In continuous mode, the CH_4 content in the biogas could be enriched through substrate
735 addition and recirculation of biogas. However, a molar deficit of H_2 and CO_2 always remains
736 inside the system, considering HM utilizes a stoichiometric H_2: CO_2 ratio of 4:1. Thus,
737 through this concept, in addition to CH_4 enrichment, CO_2 deficiency in the second stage
738 reactor could be overcome. Also, a stable operation of AD could be achieved. Here, a novel
739 concept for the *in-situ* biological biogas upgradation system is proposed (labelled as
740 Hydrogenotrophic Anaerobic Biotechnological System for Enrichment of Biogas (HABSEB)
741 Technology by the authors), which is yet to be developed. This concept could make the
742 system self-sustainable rather than relying on other conventional or renewable energy

systems to produce H_2, thus playing a more significant part in techno-economics and environmental impact. The H_2 production and its utilization for *in*-situ biogas upgradation could vastly reduce the cost required for H_2 production, which is otherwise very costly [23]. The non-requirement of additional post biogas polishing facilities and lower energy requirement, a salient feature known for *in*-situ biogas upgradation as opposed to conventional biogas upgradation techniques, could also minimize the cost economics. The CO_2 capturing and conversion to CH_4 and lower energy requirement could reduce the environmental impact. Carbon neutrality is expected to be almost equivalent to or a step higher than a two-stage AD system. The techno-economics and environmental sustainability of the novel concept would also depend upon the collection and transportation of the feedstocks to the plant, biogas productivity and upgradation efficiency [170]. For this concept, the factors to be considered could be no. of first stages, H_2 dosing rate and biogas recirculation rate and associated mechanical attachments, the applicability of digestate slurry etc. Furtherance in developing the proposed concept is only possible through validated laboratory-scale to pilot-scale investigations and evaluation of its techno-economics and life cycle analysis.

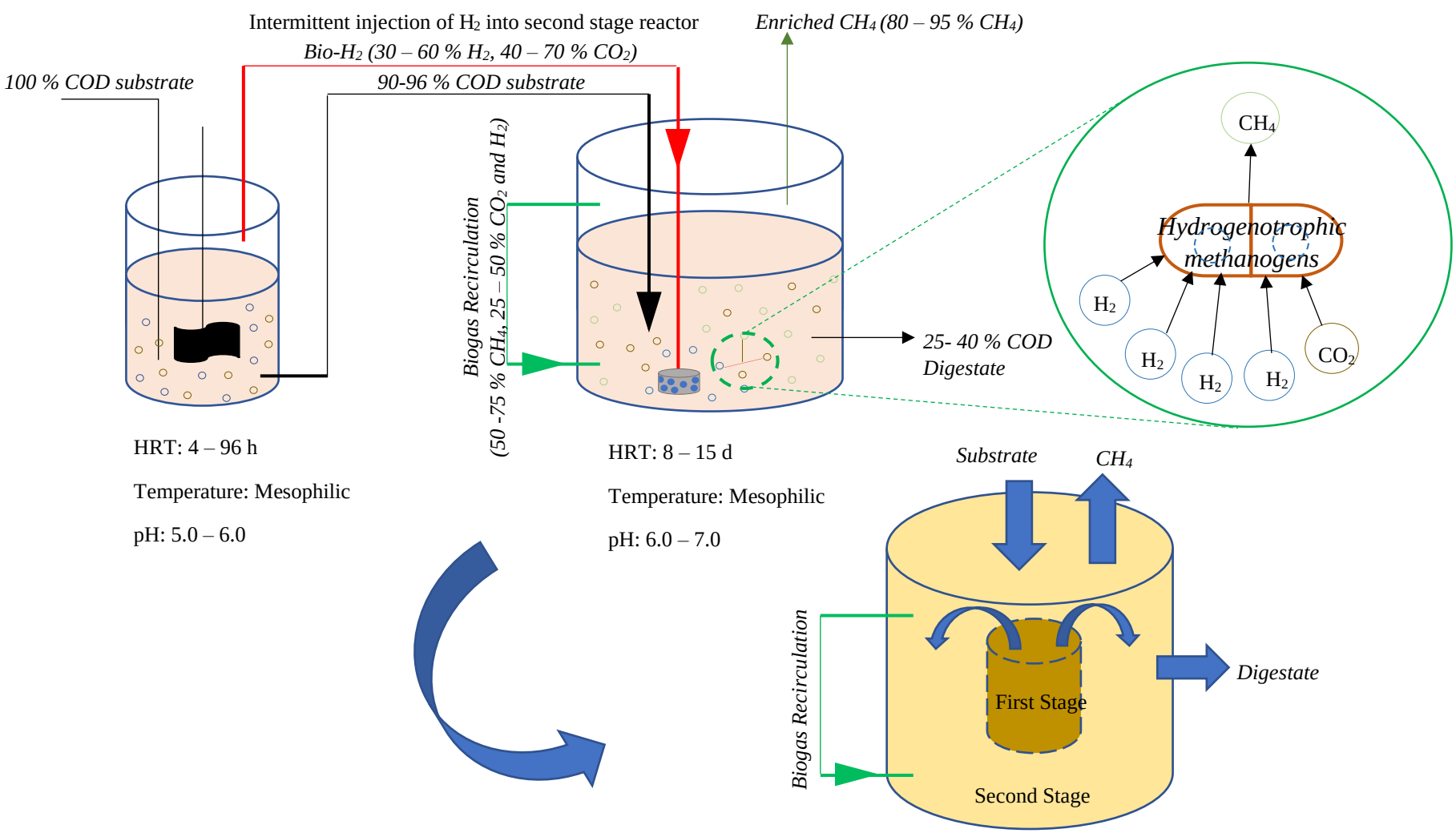

Fig. 4. The possible concept of two-stage bio-H_2 and CH_4 co-production and mass balance (conceptualized and modified from [149] and [153].

8. Conclusions

The anaerobic digestion through acetoclastic methanogenesis faces several challenges, such as high instability and lower biogas production with less CH_4 content. This article discusses the possibilities of transforming acetoclastic methanogenesis to hydrogenotrophic methanogenesis through *in-situ* biological transformation for enhanced CH_4 content in the biogas generated. This review also discusses the change in microbial population during the transformation from acetoclastic to hydrogenotrophic methanogenesis. The study further discusses the ways to achieve the concept considering an example of FW-AD. The idea is achieved through a two-stage AD system where hydrolysis to acetogenesis takes place for H_2 production in the first stage, and in the second stage, methanogenesis exists for CH_4 production. This novel concept could up bring the possible challenges faced in conventional acetoclastic methanogenesis. In addition, further research evaluations are required to apply this conceptual system to be evaluated in terms of reactor design and the optimization of process parameters, techno-economics, and life cycle analysis for the upscaling of the system for mainstream application.

Finally, based on the overall review, the following summarizes the key findings relevant to the development of microbial biogas upgradation system:

- Although this study emphasized upon food waste, all the high organic substrates or co-digestion are suitable for hydrogen-assisted microbial pathways optimizing the process parameters
- Optimization of process parameters such as pH, VFAs, NH_3, HRT could improve the CH_4 content and microbial activity irrespective of the operating temperature
- However, the selection of hydrogen utilizing microbial pathway (either SAO-HM or HA-AM) is highly dependent upon operating temperature with comparable CH_4 yield

- Solubility of H_2 gas in aqueous solution and gas-liquid mass transfer are the main challenges faced for mainstream application of hydrogen assisted microbial methane enrichment
- Biological H_2 production through dark fermentation could reduce the economic burden and energy requirement for hydrogen-based microbial biogas upgradation
- A two-stage AD strategy could be the best strategy that could suitably upgrade the existing AD systems to hydrogen assisted pathways
- The selection of a viable pretreatment method for the seed sludge is vital for producing maximum H_2 yield from the substrate in the first stage reactor.
- Long term operation of the system could acclimatize the microbiology inside the hydrogen-assisted AD system for enhanced process performance
- The application of biochar in the second stage reactor could be vital in increasing the buffering capacity, surface area, enhancing the microbial abundance and boosting the H_2 utilization.
- Syntrophic activities between acetoclastic and hydrogenotrophic methanogens, SAOs and homoacetogens are essential for the practical applicability of the proposed HABSEB system.

Acknowledgements

Authors, H.S., R.C. and V.K.V. would like to acknowledge the funding received from UK-India Education and Research Initiative (UKIERI) and Department of Science and Technology (DST) India through the grant agreement IND/CONT/GA/18-19/16, which made their participation in this research possible. The authors, R.C and K.L.K. acknowledge the funding received from the Indo-Hungarian Joint Project by the Department of Science and

Technology (DST) India through “Hydrogenotrophic Anaerobic Biotechnological System for Enrichment of Biogas (HABSEB) Technology for Power and Vehicular Fuel Applications” (DST/INT>HUN/P-21/2020(G). K.L.K. also received support from the Hungarian project 2020-3.1.2-ZFR-KVG, Circular bioenergy production via linking biogas generation with the P2G reaction”. The authors, D’ Silva and Isha are thankful to Indian Institute of Technology Delhi for their research fellowship.

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