

Critical factors influencing biohythane production from research to commercialization

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Highlights

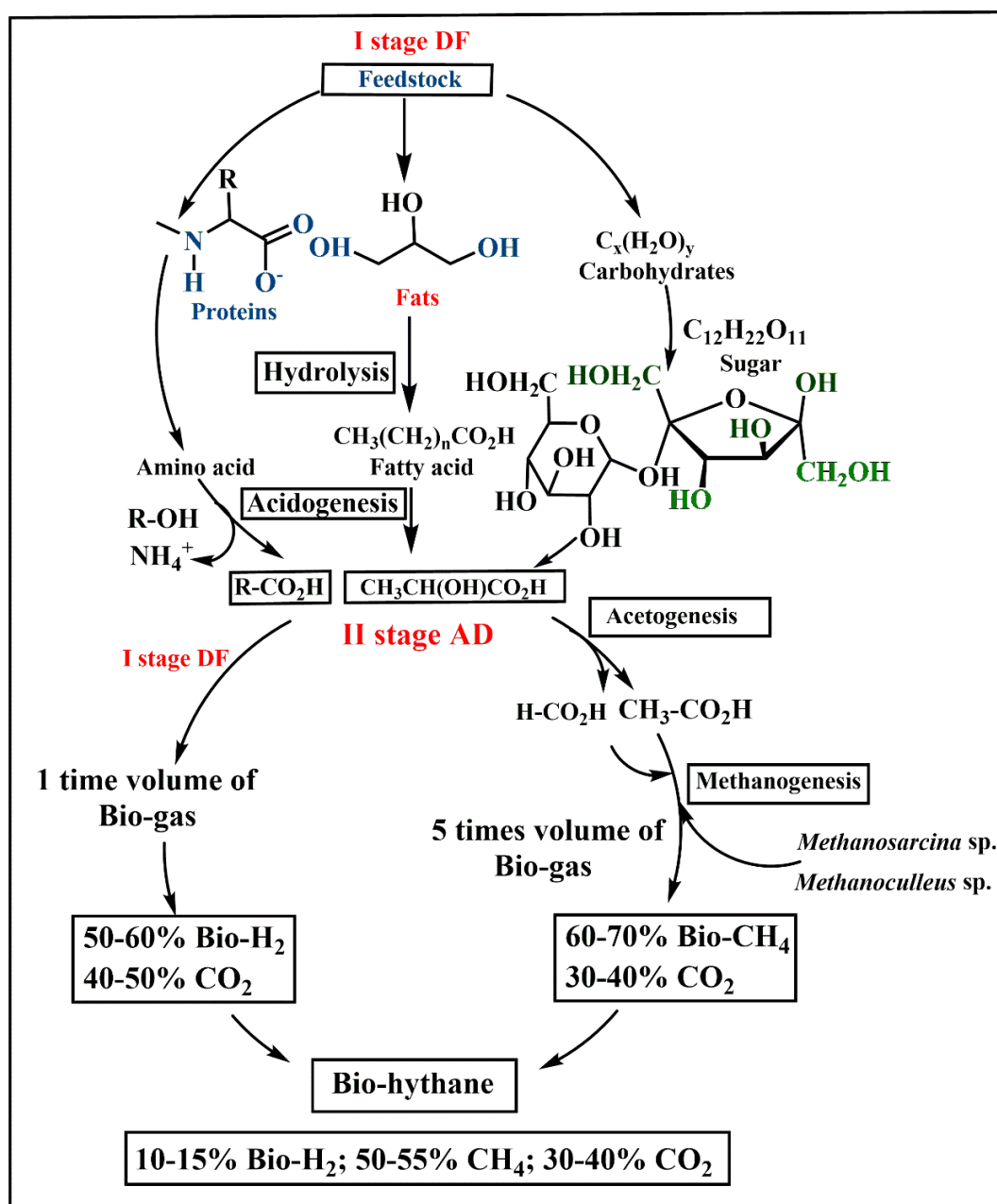
- Bio–hythane integration in waste management systems
- Effect of interaction among various influential parameters
- Futuristic approaches in molecular /genetic tools
- Application of MEC system
- SDG approach to assess the scenarios over a ten-year period (2023–2033)

List of Abbreviation

<i>Acetyl Co-enzyme A</i>	: <i>Acetyl-CoA</i>
Acyl-homoserine lactones	: AHLs
Ammonia	: NH ₃
Anaerobic digestion	: AD
Adenosine triphosphate	: ATP
Adenosine triphosphatase	: ATPase
Bicarbonate	: HCO ₃ ⁻
Business As Usual	: BAU
Bio-electrochemical system	: BES
Bio-hydrogen	: Bio-H ₂
Bio-CH ₄ production rate	: BMPR
Bio-methane	: Bio-CH ₄
Coenzyme B (N-7-mercaptoheptanoyl-O-phospho-l-threonine	: B(HS-CoB)
Carbon monoxide	: CO
Carbon dioxide	: CO ₂
Chemical oxygen demand	: COD
Carbon-to-nitrogen ratio	: C/N ratio
Carboxymethyl cellulose	: CMC
Compressed natural Gas	: CNG
<i>Clostridium butyricum</i>	: <i>C. butyricum</i>
<i>Clostridium pasteurianum</i>	: <i>C. pasteurianum</i>
Continuous stirred-tank reactor	: CSTR reactor
Dark fermentation	: DF
Direct interspecies electron transfer	: DIET
Electroactive microbes	: EMs
Elemental phosphorous	: P
<i>Enterobacter aerogenes</i>	: <i>E. aerogenes</i>
<i>Escherichia coli</i>	: <i>E. coli</i>
Ferrosoferric oxide	: Fe ₃ O ₄
Oxidized ferredoxin	: Fd _{ox}
Reduced ferredoxin	: Fd _{red}
Enriched mixed culture	: EMC
Evolution of carbohydrate metabolism	: EMPM
<i>Formate hydrogen lyase</i>	: FHL
Foodwastes	: FWs
Green house gases	: GHGs
Hydrogen-to-carbon ratio	: H:C
Organic disulfide and a S-substituted coenzyme M	: Hetero-disulfide CoM-S-S-CoB
H ₂ -enriched compressed natural gas	: HCNG
Hydrogen evolution reaction	: HER
Hydroxyl-methyl-furfural	: HMF
Hydraulic retention time	: HRT
Hydrogenase isoenzymes	: <i>hya</i> , <i>hyb</i> , <i>hyc</i> , <i>hyd</i> and <i>hyf</i>
Electron donor coenzyme B	: <i>HS-CoB</i>

H ₂ partial pressure	: HPR
<i>Lactate dehydrogenase</i>	: ldh
Life cycle evaluation	: LCE
Lignocellulosic biomass	: LCB
Methyl-coenzyme M reductase (methylthioethane sulfonate)	: MCM (CH ₃ -SCoM)
Methyl-coenzyme m-reductases	: MCMR
Microbial electrolysis cells	: MECs
Microbial fuel cell	: MFC
N-methyl-N-nitro-N-nitrosoguanidine	: NTG
Nicotinamide adenine dinucleotide	: NADH
Oxidized form of Nicotinamide adenine dinucleotide	: NAD ⁺
Organic fraction of municipal solid waste	: OFMSW
Organic loading rate	: OLR
Olive mill wastewater	: OMW
<i>Pyruvate formate lyase</i>	: PFL
Palm oil mill effluent	: POME
Pressure swing absorption	: PSA
Solid carbon dioxide pretreatment of aerobic granular sludge	: S-CO ₂ /AGS
Sustainable development goals	: SDG
Siphon-driven self-agitated anaerobic reactor	: SDSAR
Solids retention time	: SRT
United nations	: UNs
Up-flow anaerobic sludge blanket reactor	: UASB
Volatile fatty acids	: VFAs
Total solids	: TS
equivalent per megajoule	: eqMJ ⁻¹
gram/litre	: g/L
active reactor volume	: g COD L ⁻¹ d ⁻¹
Proton / Hydrogen ion	: H ⁺
Water	: H ₂ O
Kelvin	: K
1000 Euros	: k€
Megapascal	: MPa
Metric ton	: MT
Oxides of nitrogen	: NO _x
ΔG	: Change in Free energy

Graphical Abstract



Abstract

The shift from fossil-derived energy to clean, renewable sources has accelerated due to the demand for sustainable and commercially viable energy solutions. Another possible clean fuel is biohythane, also known as HCNG, which is a blend of bio-CH₄ and bio-H₂ with a concentration of 10 and 30% v/v, respectively. The chemical industries have eventually been utilising bio-CH₄ and bio-H₂ extensively because of their high calorific values of 143 and 55 kJ g⁻¹, respectively. The bio-H₂ and bio-CH₄ have been emerged as promising green energy carriers, offering a broad range of applications in chemical industries, owing to their high calorific value, renewability, and CO₂ neutrality. In the pursuit of zero-emission technologies to mitigate the global warming, biohythane is gaining attraction as a potential future fuel. This study explores biohythane production through a sequential two-stage process that converts organic wastes into bio-H₂ and subsequently into bio-CH₄, offering an ideal pathway for sustainable biohythane generation. Further, this study provides an overview of key developments and applications in two-stage microbial synthesis of bio-CH₄ and bio-H₂, alongside insights into the demand, supply, and current global status of biohythane production. This study delves into the acidogenic phase, where bio-H₂ is produced, and the methanogenic phase, which yields bio-CH₄. While detailing the biochemistry, critical factors, challenges, and limitations of an integrated bio-hythane production system are discussed. Finally, the strategies for enhancing bio-H₂ and biohythane production and an outlook on their commercialization potential are discussed.

Keywords: *Bio-H₂; Bio-CH₄; Biohythane two-stage path; BES systems; Critical factors*

1. Introduction

In the current time, we are over dependent on fossil fuels as source of energy. The fossil fuels are non-renewable and finite and at brink of extinction. Their overuse has negative impact on environment and raised serious global concern including causing a significant increment in carbon emission (Agrawal et al. 2023). The situation is going to aggravate with increase in energy demand due to continuously growing human population and urbanization. It is predicted that global energy demand will rise from 564 EJ in 2020 to 670 EJ in 2030 and double by 2050 (Dangol et al. 2022; Kim et al. 2022). The UNs expect an increase in population by almost 50% in the next 20 years, thereby requirement of the energy also said to become upturn (Sanjay Kumar et al., 2021). The gravity of situation can be understood by the fact that over 130 countries have signed the pact of net-zero coalition to cut down on global warming and achieve goal of zero emissions (Dangol et al. 2022; Sasidhar et al. 2022). All these has resulted in a paradigm shift towards renewable, green and environmentally friendly fuels. One of the solutions to fulfil the energy demand is bio-based renewable source of energy. The combination of dark fermentation (DF) and anaerobic digestion (AD) can produce a renewable biofuel known as biohythane which is a mixture of two energy carriers, bio-hydrogen (H_2) and bio-methane (CH_4), produced from organic wastes. The sequential production of both bio- H_2 and bio- CH_4 together (biohythane / HCNG) from such sources is being paid attention as it possesses the socio-economic benefits from the recent past, owing to its functional stability, efficient yield of energy and greener by-products (Ta et al., 2020). The biohythane has become a viable energy source for the shift to a hydrogen-based economy by means of several microbes mainly like *Bacillus*, *Methanosarcina*, *Limnochordia*, *Syntrophomonas*, *Lentimicrobium*, *Hydrogenispora*, etc., from the biomass wastes during anaerobic digestion (Alavi-Borazjani et al., 2024; Liu et al., 2025; Yapıcıoğlu 2025). To attain the carbon neutrality for the consequences of global warming, the biohythane mixture must contain 10–30% of bio-hydrogen that increases the thermal efficiency and lowers the greenhouse gas emissions (Özmihçı et al., 2025).

Biohythane, a high-grade fuel which is better than methane in terms of easier ignition, flammability and flame speed. The carbon emission for methane and hydrogen production is less in comparison to the chemical route. Biohythane obtained from AD of waste biogenic residues is emerging as a potential biofuel (Roy and Das, 2016; Bolzonella et al. 2019; Dangol et al. 2022). Bio-hythane has the potential to significantly increase the economic viability and boost-up the

energy recovery by about 08–43%. The biohythane has been used directly as a commercial vehicle fuel in many countries, including China (Beijing Hythane Bus Project), India, California, Canada (Montreal Hythane Bus Project), and well-known automakers like Volvo and Fiat, among others, because it can minimize the CO emissions by up to 50% (Rashmi Ira et al., 2024). Recently, the researchers have recommended production of valuable bio-fuels including bio-diesel, bio-ethanol, bio-H₂ and bio-CH₄ from the waste biomass-sources like *Moringa oifera* (K.B. Sasidhar et al., 2022), chicken fats (Ntaikou et al., 2010), coffee spent grounds and coffee silverskin (Mirzoyan et al., 2022; Vanyan et al., 2022; 2024) and some other edible oil (Veluswamy et al., 2019). In that way, it is more suitable that a sequential production of both bio-H₂ and bio-CH₄ together (biohythane) by treating the solid wastes. The biohythane is a blend of bio-hydrogen and bio-methane of about 10–25% / 05–7% by volume / by energy (Sanjay Kumar et al., 2021), respectively. In comparison to the conventional solid waste valorisation by means of composting / landfilling and incineration, an eco-friendly disposal *i.e.*, AD has been adopted as it provides revenue *via* production of energy besides its stable treatment pathway (Uddin and Wright, 2023; Xu et al., 2018).

Wastewater has widely been recognized as a valuable source for bio-hythane production *via* AD, generating bioenergy and other value-added products (Mahata et al., 2023; Mahmud et al., 2021; Mutsvene et al., 2023). Angelidaki et al., (2003) identified several potential applications of AD, including (i) activated and secondary clarifier sludge from wastewater treatment plants, (ii) agro-industrial wastewater, (iii) food industry waste, (iv) agricultural and livestock waste, and (v) the OFMSW. Additionally, microbial populations from waste sources have been effectively used to degrade organic fractions from both solid and liquid waste streams, yielding biogases, power, fuels, and other energy-rich bio-products *via* the AD process (Lastella et al., 2002; Wainaina et al., 2019). Emphasis is also placed on recovering organic carbon in the form of volatile fatty acids (VFAs) and bio-H₂, which enhance the economic and environmental value of the process (Goveas et al., 2023). Bio-H₂ is a green and sustainable fuel as its combustion process results in water vapours formation only and it possesses an energy density of about 122–142 kJ / g (Liu et al., 2013; Marone et al., 2014; Trchounian et al., 2017a). Further, the co-production of bio-H₂, and bio-CH₄, by means of a two-stage AD process has currently been growing as it is a viable to the future society with zero carbon emission (Cheng et al., 2016a, 2016b; Giuliano et al., 2014; Khan et al., 2018a; Lee and Chung, 2010; Liu et al., 2013; Rena et al., 2020; Shanmugam et al., 2021a;

Wainaina et al., 2019; Xiao et al., 2019; Yu et al., 2022). A schematic representation (Fig. 1) shows the production sequentially by bio-H₂ and bio-CH₄ from solid organic wastes *via* AD process, which involves usually two-stages.

Though bio-CH₄ is a cleaner fuel when compared to the conventional ones, it has a very poor range of lean flammability (Bauer, 2001a), which lead to a poor efficiency on combustion (Liu et al., 2013). Thereby, a suitable combination of tracer amounts of bio-hydrogen significantly improves the flammability range of bio-CH₄ to enhance fuel efficiency and biohythane applicability in Spark-Ignition (SI) engines too (Bauer, 2001a, 2001b; MA, 2008). In this view, this study firstly summarises the works and applications carried out in the field of two-stage sequential H₂ and CH₄ production *via* biological route. Besides, it highlights on demand and supply, current status of global production in biohythane. Additionally, it explains the production of bio-H₂ from agro-industrial organic waste, acidogenic phase as well as BES systems and bio-CH₄ by methanogenic phase. The two-stage and integrated biohythane production system, its biochemistry, challenges and limitations *via* nature of biocatalysts, enriched mixed culture, operational critical factors / parameters that affect biohythane production *viz.* pH, temperature, metabolic activities and storage/dispensing stations have been discussed. Further, the strategies to improve biohythane production by means of reactor design, bio-stimulators, co-digestion, pretreatments, molecular/genetic tools and microbial electrolysis as well as its future outlook are discussed. Finally, as a novel approach, this study will assess the potential of biohythane as a clean fuel, based on a cost-benefit analysis and environmental impact with respect to other fuels towards its commercialization.

#Fig. 1

A thorough review of the literature revealed that, mainly from the perspectives of bio-energy yield, operational parameters, and both economic as well as environmental evaluations, there is not a single previous review on the comparative evaluation of the three acidogenic processes for the valorisation of organic wastes (Abdur Rawoof et al., 2021; Velusamy Mozhiarasi et al., 2023). To the best of the authors knowledge, no comparative review studies have been conducted, and no comparative analysis of the three involved acidogenic pathways for the feedstocks has been investigated. Therefore, a review of comparative studies regarding yield, reactor setup, economic and environmental sustainability, and other factors is required for the bio-hhythane production from the organic wastes. Thereby, providing a systematic review of

comparative studies on the production of bio-hythane *via* anaerobic fermentation processes, this study aimed to close the existing research gaps.

1.1. Bio-H₂ / Bio-CH₄ (Bio-hythane) production demand and supply

Bio-hythane has highly been raised as a clean vehicle fuel, since 1980. Though, both bio-H₂ and bio-CH₄ has been a notable greener fuel when compared to the petro-derived diesel / gasoline, limited by means of certain facts such as higher ignition temperature, narrow range of flammability and slow-burning (Sanjay Kumar et al., 2021). Thereby, the overall output for CNG vehicles possess poor combustion efficiency and so there is a necessity of high ignition energy. When DF and AD are combined, both the bio-H₂ and bio-CH₄ can be produced simultaneously. Improved reactor configurations, different substrate pretreatment techniques (physical, chemical, and biological) to speed up the hydrolysis, and methods to control formation of inhibitory agents and resistant substances like HMF and furfural from high-temperature pretreatment are the examples of advancements in DF and AD technologies. Significant advancements have also been made in co-digestion techniques, microbial consortia, and DIET pathway (Hassan Mohammed Ali et al., 2024). Further, the bio-H₂ perfectly fulfils the fading points of bio-methane. Thus, the mixture of both bio-H₂ and bio-CH₄ increases the H:C ratio, thereby it has been observed that there is a reduction on GHGs emissions along with enhanced fuel as well as heat efficiency, flame speed and shorter combustion period (David et al., 2019). Beyond, biohythane possesses some advantages, which can eventually be considered as the research keys for optimisation of vehicle fuel efficiency in its production as well as industrial utilization (Veluswamy et al., 2019) *i.e.*, combustion characteristics, ignition performance and emissions of GHGs (Sanjay Kumar et al., 2021). The production of both the fuels, separately from the fossil-derived sources is generally unsustainable and energy- and cost-intensive. The accentuated benefits of the combination of bio-H₂ and bio-CH₄ as vehicle fuel are (i) low flammability of bio-methane that limits the fuel efficiency and capability of bio-hydrogen to increase the range of lean flammability, and (ii) stimulating capability of bio-H₂ to quicken the combustion process of bio-methane. At low temperature, its capability as a reducing agent can be used for effective catalysis.

1.2. SDG approach

The SDG approach to assess the scenarios over a ten-year period (2023–2033), according to quantitative findings, sustainability under the BAU and optimistic scenario achieves 78.90%, improvement which is deemed "Sustainable," while the moderate scenario improves to 71.62%,

which is categorized as Sufficiently Sustainable. Such findings highlight how strategic policies, like government subsidies, projects, and community-based training programs, have a transformative effect and lead to a 63.1% improvement in sustainability from BAU to optimistic scenarios (Sinsuw et al., 2025). Additionally, these measures enhance the waste management to boost-up the economic resilience, and lower CO₂ emissions. The innovative aspect of this study is how it provides scalable and practical policy suggestions by combining socio-economic and environmental aspects within a dynamic SD framework. Furthermore, bio-H₂ improves the internal engine performance that frequently fed by the fossil-derived ones and thereby the biohythane production is related to knock-resistance (Sanjay Kumar et al., 2021). In comparison to bio-CH₄, the bio-H₂ ignition energy is somewhat low, henceforth it helps the latter to burn better. In this way, biohythane have obviously been attracted the sector of automobiles, predominantly to the highlights on such features. For instance, the biohythane vehicles has been industrialized by Toyota, one of the car manufacturers who comes mainly with the feature of frequent consumption of energy. Above all, it is a persuasive intermediate for the production of value-added compounds as well as liquid fuels.

2. Biohythane production

The biohythane production mainly involves an AD process by means of one-stage (one-reactor) / two-stage AD (two-reactors) process to synthesize the bio-gas (Bertasini et al., 2024; Fanfoni et al., 2024; Sillero et al., 2024). Actually, it occurs *via* the hydrolytic separation and two-stage acidogenic AD processes, as a result of DF reaction (Roy and Das, 2016). The released biogas is enriched with bio-H₂ and bio-CH₄, subsequently after both the acetogenic and methanogenic phases. Within a favourable temperature and pH, bio-hydrogen can be produced through DF by certain anaerobes like *Bacillus*, *Enterobacter*, *Thermoanaerobacterium* sp., *Clostridium* sp., *via* chain microbial processes (Battista et al., 2016, Micolucci et al., 2016). Mostly, both the *Methanococcus* and *Methanosarcina barkeri* microbes are more sensitive and can assist a complete microbial bio-methanation process. The corresponding activities among the microorganisms make the methanogens to utilize bio-hydrogen, produced at the acetogenic phase, meanwhile with suitable anaerobic conditions, there might be the production of both bio-methane and CO₂. Thereby, the biohythane generation includes dark fermentative as well as acetogenic phases and each can obviously have regulated by means of specific microbes with varying reaction conditions like a balanced temperature, HRT, nutrients, OLR, partial pressure and pH (Battista et

al., 2016). The metabolic activities need the micronutrients at a low level to access the two-stage AD process (Micolucci et al., 2016). Finally, the process requires vacuuming of hydrogen and methane for upgrading the phases to eliminate the acidic compounds, ammonia, CO₂, siloxanes, and water from the generated bio-gases by means of certain preferred techniques like chemical (22%) and, water scrubbing (41%) / PSA (21%). Recently, the studies show that 1 / 10th of the upgradation can be carried out by membrane separation technique.

The implementation of innovative techniques, which is due to growing interest towards the production of bio-hythane obviously leads to an advance development in its production and quality, as well. In a previous study, authors (Sarkar and Venkata Mohan, 2017a) established an optimization in the pretreatment process by injecting about 400 L of bimolecular oxygen for 60 min on Food waste substrate (FWs). Thereby, the suppressed methanogens by the presence of air eventually reduce the effective conversion of VFAs into bio-CH₄ that is not desirable in the DF process for the generation of bio-H₂. Furthermore, the pre-aeration assists the hydrolytic process of the complex FWs into simple molecules (David et al., 2019). Such an operation by pre-aeration allows an increase in the amount of bio-H₂ and bio-CH₄ of about 97 and 10%, respectively in addition to the production of VFAs. The disadvantage is the possibility towards infiltration of oxygen for the next methanogenic phase of AD process to cause inhibition. Here, optimization of the process of bio-hythane can be achieved by altering the mixing procedure, HRT, and temperature. The thermophilic circumstances quicken kinetics of microbial activities to permit the reduction in HRT, reactor volume and, inevitably, the costs on installations. It is significant to declare a prolong HRT requirement for evasion of the methanogens. Similarly, various experiences with thermophilic processes in lab-scale for the sewage sludge treatment could yield low bio-methane for about 0.15 m³ /kg VS, fed in accordance to the HRT values in 8 to 9 days (Cavinato et al., 2011). Mamimin et al., (Mamimin et al., 2015) showed some interruptions in the mixing process and planned to mix them 2h, earlier to the substrates feeding. It makes the methanogens functional to mechanically produce stress, here they may grow up better and function well. Such a technique of processing eventually reimburses the methanogens flop, because of the lower HRT values. Further, Maminin et al., (Mamimin et al., 2015) achieved a bio-hythane production of about 350 L/kg VS, along with some bio-methane by about 65% v/v. Lastly, an additional approach towards an advance production of biohythane to improve the metabolic activities in the presence of micro-organisms by the addition of certain metal ions like Cu²⁺, Fe²⁺, Mg²⁺, P³⁻, Zn²⁺,

etc. The presence of such metal ions can act as various enzymes co-factors, which is significant to the fermentation path to intensify the amount of biohydrothane and improve its quality, as well (Battista et al., 2016; Mishra et al., 2017). Table 3 shows various microbiome involved in biohydrothane production. Further, Fig. 2 depicts the combination of acidogenesis and methanogenesis for the production of bio-H₂ and bio-CH₄ (biohydrothane).

#Fig. 2

2.1. Bio-H₂ production system-Acidogenic phase

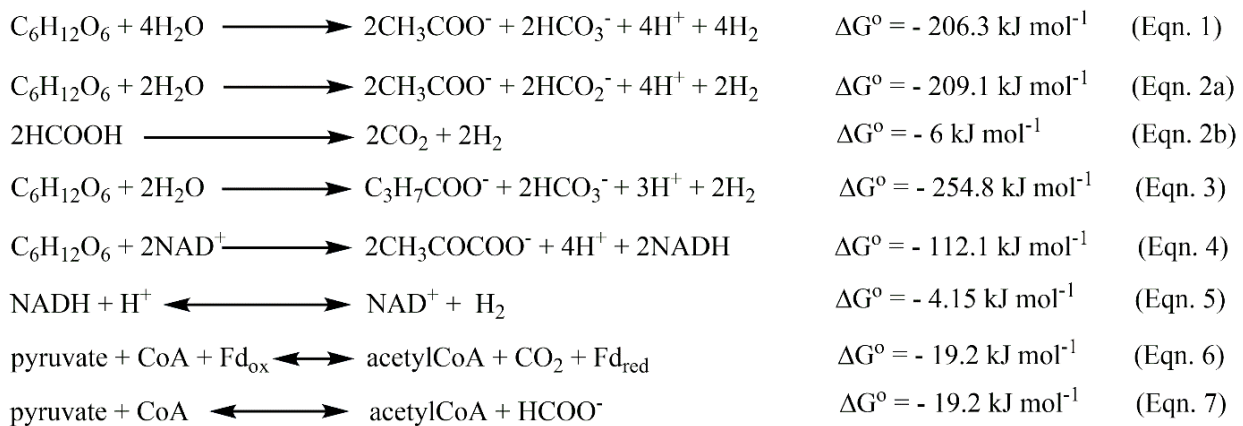
The DF processes mainly for generation of bio-H₂ can be achieved strictly *via* anaerobic / facultative anaerobic bacteria (Kora et al., 2023; Ntaikou et al., 2010; Sarkar and Venkata Mohan, 2017b). Both the theoretical and the estimated yield values towards fermentative hydrogen and the biotransformation of glucose respectively not only depend on the acetate, but also on dissimilar organic moieties certainly like carbohydrates, lipids, proteins, sugars, etc., which all can obviously be utilized as reference materials (Dahiya et al., 2023; Ram et al., 2023; Sganzerla et al., 2023). The reaction [Eqn. (1)] (Alavi-Borazjani et al., 2024; Ntaikou et al., 2010) implies a maximum theoretical bio-H₂ yield from the fermentation process of glucose is 4 mol H₂ per mol of the glucose that was consumed. It can be achieved by two steps *viz.* the glucose fermentation to acetate and formate [Eqns. (2a) and (2b)] (Alavi-Borazjani et al., 2024; Ntaikou et al., 2010). If the product will be butyrate, then the maximum bio-H₂ theoretical yield is about 2 mol H₂ / mol of the utilized glucose moieties [Eqn. (3)] (Ntaikou et al., 2010). Fundamentally, the step that is common to all the above reactions is the glucose metabolism towards formation of the pyruvate units [Eqn. (4)], by which 2 moles of bio-H₂ can theoretically be produced *via* the regeneration of NADH (Eqn. 5) that was subsequently generated in this process. While, by most of the bacterial metabolism of glucose into pyruvate is frequently being carried out by means of the EMP pathway (Ntaikou et al., 2010).

However, the *acetyl-CoA* plays a key enzymatic co-factor system for production bio-H₂ from organic matters that can further be generated by pyruvate. This *acetyl-CoA* system can mostly be generated by the reaction, [Eqns. (6) or (7)] further it can be catalyzed by *pyruvate-ferredoxin oxidoreductase*, here *ferredoxin* coenzyme acts as electron acceptor (Ntaikou et al., 2010). Mostly, the anaerobic as well as facultative anaerobic bacteria and cyanobacteria can synthesize this enzyme, in which *acetyl-CoA* system may be metabolized further to acetate / butyrate. In both reaction routes, the re-oxidation process can obviously produce one mole of

ferredoxin and hydrogen by means of the enzyme *hydrogenase* (Ntaikou et al., 2010). In certain metabolic routes of glycolysis, one mole of H₂ / mol of the consumed glucose is produced while acetate is the sole product. However, while butyrate is the product, the glycolysis of NADH (oxidation of glucose) actually occurs *via* oxidation of *acetoacetyl-CoA* system, thereby the final yield H₂ is 2 mol / mol of the consumed glucose. A schematic diagram of bio-H₂ production system with by-products by means of glycolysis is depicted in Fig. 3.

#Fig. 3.

Besides, a concurrent production of acetate and butyrate can be observed often, which mostly depends on the (i) culture and (ii) microorganisms, here the yield of bio-H₂ falls in between 2 and 4 mol / mol of the consumed glucose. These reaction pathways are the characteristics of some clostridia like *C. pasteurianum* and *C. butyricum* (Ntaikou et al., 2010). Then, the generation of *acetyl-CoA* [Eqn. (7)] (Alavi-Borazjani et al., 2024) obviously leads to the formation of formate, simultaneously and this reaction can eventually be catalysed by means of the *pyruvate-formate lyase* enzyme, under anaerobic conditions. It is a typical metabolism of some *enterobacterial* species, like *E. aerogenes* and *E. coli*, etc.



Besides, some clostridia have also been reported for the existence of *pyruvate-formate lyase* system (Ntaikou et al., 2010). Some other glucose fermentation products besides acetate, butyrate and formate are predominantly 2,3-butanediol, lactate, propionate, and succinate. Such products can directly be derived from the pyruvates, further ethanol, butanol and isopropanol can obviously have obtained from the *acetyl-CoA* metabolism (Trchounain et al., 2017b). All the above-mentioned metabolites during dark fermentative production, is not at all followed by the generation of bio-H₂. Thereby, these products have been characterized as undesired by-products,

which are often obtained from the mixed metabolic bacteria / mixed cultures fermentation (Kirkpatrick et al., 2001).

2.2. Bio-H₂ production in BES systems

A sustainable technology namely, BES allows the bio-H₂ and bio-CH₄ production from the biomass materials that represents the MECs, further it leads to a modified formulation of MFC (Gkotsis et al., 2023; Phan et al., 2023; Riaz and Park, 2022; Sarkar et al., 2023a; Tang, 2021). The MECs design and configuration impacts greatly the rate of bio-H₂ yield as well as the current density (Bora et al., 2022). MECs include a range of designs, which are ranging from single-chambered (membrane-less) to dual-chambered (with various membranes) configurations. Moreover generally, the architecture of MECs are designed and operated in other forms of configurations mainly like cubic, rectangular, tubular, *etc* (Sarkar et al., 2023a). Here, the renewable energy / value-added products can be obtained *via* transformation of renewable carbon from the lignocellulosic bio-wastes / dairy, distillery, sewage, swine wastewaters (Chaurasia and Mondal, 2022). In MFCs, the cathode is placed inside of the cathode chamber, and not exposed to air / O₂. Here, applied voltage facilitates the HER *via* the combination of electrons and protons. In the anode, bacteria consume the substrates and release the reduced equivalents, here the voltage produced is found to be about -0.3 V, whereas the HER needs -0.41 V. Though, about 0.25 to -0.80 V applied voltage is required to come across the production of hydrogen for HER in the MEC system (Sarkar et al., 2023a). The major benefits of such a production of bio-H₂ using MEC systems is the absorption / utilization of low energy and high-purity of the products as compared to the electrolysis of water (Rousseau et al., 2020). The substantial yields using MECs for the production of bio-H₂ can be carried by means of both batch and continuous modes (Sarkar et al., 2023a). In the economic point of view, membrane less single-chambered MECs are comparatively valuable to attain the higher rates of production (Khan et al., 2017). Avoiding the usage of membranes in single-chambered MECs leads to limit the resistance for density of current, thus there observed a further enhancement towards the yield of bio-H₂. The research span for several years exemplifies the importance of MEC functions using a single chambered reactor with continuous mode towards the yield of high bio-H₂ production and rates with low-price (Sarkar et al., 2023a, 2022).

2.3. Bio-CH₄ system-methanogenic phase

The production of bio-CH₄ by means of a two-stage AD process for bio-hythane generation also represents the best sustainable alternative to an increase in the overall yield. The DF constitutes the VFAs substrates by the endothermic reactions, which yield acetate as the major product (Sanjay Kumar et al., 2021). Whereas the dark fermentative acidogenic bacteria can be inhibited by means of a high concentration of hydrogen, on the contrary, the microorganisms that can produce bio-CH₄ only survive at a high partial pressure of hydrogen. Under strict anaerobic conditions, the two microorganisms can co-operate, thereby the methanogenic ones utilize effectively the produced hydrogen in the acetogenic phase (Korres et al., 2010). Consequently, at its low concentration, there forms both the bio-CH₄ and CO₂. This phase is the limiting control step for the two-stage AD process. The production of bio-CH₄ occurs by means of two reaction pathways *viz* hydrogenotrophic and acetoclastic paths. In the first process, the hydrogenotrophic bacteria oxidize the hydrogen into bio-CH₄ [Eqn. 8]. In the acetoclastic path, there occurs the reduction of acetate into bio-CH₄ and CO₂ [Eqn. 9] (Friedrich, 2005).

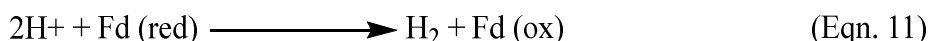


Even though, about 70% of the bio-CH₄ production actually follows this reaction path, while both the two different processes are crucial to the AD process. At the end of the second path of AD process, a blend of gaseous products H₂:CH₄:CO₂, *i.e.*, biohythane is produced. The volumetric compositions of such products are frequently about 10 %:60 %:30 %, respectively that can facilitate the direct utilization of biohythane as bio-fuel (Kumari and Das, 2019). Anyhow, an upgrade stage has been recommendable because of the existence of the finally formed corrosive compounds. Such an upgradation process can obviously have utilized to eliminate certain products like acid compounds, CO₂, H₂O, NH₃, and siloxanes from the biogases by means of water scrubbing (41%), consequently followed *via* chemical scrubbing (22%), and finally by pressure swing adsorption (21%). Furthermore, the membranistic separation has been received considerable attention from the last few years, and it has been utilized for upgradation of 10 % units (Sanjay Kumar et al., 2021).

2.4. Two-stage and integrated biohythane production

The synthetic process of hydrogen-methane blend, bio-hythane involves various types of microorganisms that somewhat can function synergistically. After the hydrolysis of complex

substrates into simple sugars, they can be metabolized by means of glycolytic pathway to pyruvate *via* DF. Thereafter, the microorganisms could receive energy in the form of ATP for the conversion of pyruvate to acetate as well as butyrate, simultaneously a secondary product as hydrogen. Especially, the mechanistic pathways may differ consequently if the microorganisms are obligate (*Clostridium* sp.) / facultative anaerobes (*Enterobacter* sp., *E. coli*). Further, the obligate ones usually follow the reactions, which are given in Eqns. (10) and (11) (Alavi-Borazjani et al., 2024).



The reduction of ferredoxin yields *acetyl-CoA* *via* oxidation of pyruvate in the presence of *pyruvate-ferredoxin oxidoreductase* enzymatic systems [Eqn. 10] (Cremonez et al., 2021). At that moment, further the oxidation of Fd by means of *[Fe-Fe] hydrogenase* can produce hydrogen [Eqn. 11]. As an alternative, the reactions by the facultative anaerobes might be catalyzed by PFL enzymatic system that obviously supports the liberation of *acetyl-CoA* and formate [Eqn. 12], after the conversion into both CO₂ and H₂ *via* FHL [Eqn. 13].

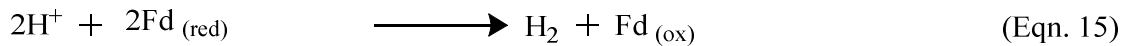


E. coli produce bio-H₂ *via* special enzymes named H_{yd} (Trchounian et al., 2012; Peters et al., 2015). It has four membranes bound H_{yd} enzymes which depending on external conditions such as pH, sole or mixture of carbon sources and their concentrations can work either in H₂ production or oxidation mode. H_{yd}-3 with formate dehydrogenase H forms FHL-1 complex (Peters and Sargent, 2023) and H_{yd}-4 with FDH-H forms second FHL-2 complex (Trchounian 2012). Particularly it was shown that H_{yd}-3 and H_{yd}-4 are working towards H₂ production during glucose (Maeda et al., 2008; Petrosyan et al., 2020) or mixed carbon sources fermentation (Mirzoyan et al., 2018) while H_{yd}-1 and H_{yd}-2 produce H₂ during glycerol fermentation (Trchounian and Trchounian, 2009). It is important to note that H_{yd} producing enzymes not only just produce H₂ but they are important complexes for balancing bioenergetics key parameters of the cell such as proton motive force (Pinske et al., 2015; Trchounian et al., 2013; Trchounian and Trchounian, 2019). It has been shown that depending on external pH and carbon sources H_{yd} enzyme differently participate in maintenance of transmembrane proton gradient and membrane potential. Moreover, the H_{yd} enzyme activity depends on carbon sources concentration which affects the H₂ production rate (Vanyan and Trchounian, 2024; Trchounian and Trchounian, 2014). H_{yd} enzymes in

cooperation with proton ATPase, key enzymes of bioenergetics relevance during fermentation, conserve energy and thus regulate proton and H₂ cycling (Trchounian, 2019). New H_{yd} producing FHL complex is suggested which acts differently at pH 5.5 and pH 7.5 (Petrosyan et al., 2020). The production of CH₄ is basically catalyzed by means of *methyl-coenzyme M reductase* that reacts with the coenzyme B (HS-CoB) as electron donor system. Thereby under an exact anaerobic condition, the formation of methane along with the hetero-disulfide CoM-S-S-CoB system in the presence of methanogenic microorganisms takes place. Accordingly, another group of methanogen is capable for the conversion of CO₂ and H₂ to CH₄. These are the CO₂ reducing methanogens, which can uptake two electrons for the conversion of CO₂ / HCO₃⁻ into CH₄. Such microorganisms can obviously use H₂ as the only source of electrons that can be attained by means of some other H₂-producing microbes. Furthermore, Table 3 shows various microbiome involved in bio-hythane production.

2.5. Biochemistry of bio-hythane production

In DF process, glucose is a common carbon rich substrate utilized for the production of bio-H₂ by various microbes (Masset et al., 2012; Zagrodnik and Duber 2024) i.e., the complex carbon rich substrates under hydrolysis can produce simple sugar units, which is further metabolized *via* a glycolytic pathway to produce pyruvate. During the DF process, the microbes gain energy in the form of ATP as well as the formed pyruvate, which will be converted into acetate and butyrate by leaving bio-H₂ as high value added by-product. In both the facultative and obligate anaerobes, the bio-H₂ production is different i.e., the process of metabolism of obligate anaerobes include the oxidation of pyruvate using pyruvate-ferredoxin oxidoreductase enzyme to produce *acetyl-CoA* with the help of Fd reduction [Eqn. 14] and then further oxidised to produce bio-H₂ with the help of Fe-Fe hydrogenase [Eqn. 15] (Alavi-Borazjani et al., 2024) as:



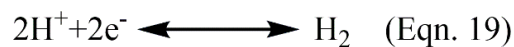
However, during the metabolism of facultative anaerobes (*E. coli* or *Enterobacter* sp.), the oxidation of pyruvate process yields formate and *acetyl-CoA*, which is further catalyzed through PFL [Eqn. 16] and the so produced formate is further split into bio-H₂ and CO₂ in the presence of *formate hydrogen lyase* [Eqn. 17] as:



Similarly, MCMR enzyme play a vital role in the production of bio-CH₄ (Methanogenesis) through DF process (Sanjay Kumar et al., 2021). In this process, the reduction of activated C1 intermediates and coenzymes are bound. The main substance MCM (*CH₃-SCoM*) reacts with *HS-CoB* to produce bio-CH₄ as well as heterodisulfide (*CoM-S-S-CoB*). Also, the MCMR *via* methanogenic organisms catalyses the whole reaction under optimum anaerobic conditions. The formed bio-H₂ and CO₂ through DF have been utilized as energy rich sources in methanogens metabolic pathway (Sanjay Kumar et al., 2021) i.e., hydrogenotrophic methanogenesis process [Eqn. 18]. During this process, the conversion of bio-H₂, CO₂ and methanofuran into N-formylmethanofuran, which is fully controlled through two enzymes namely F420-non reducing NiFe hydrogenase (electron acceptor) and N-formylmethanofuran dehydrogenase (electron donor). Generally, in the methanogenic environment, the partial pressure of H₂ is generally 1–10 Pa and their corresponding ΔG is –20 to –40 kJ / mol i.e., proving that the reaction is exergonic (thermodynamically feasible) under this circumstance (Sanjay Kumar et al., 2021).



It is notable that in *E. coli* Ni-Fe hydrogenases are encoded by *hya*, *hyb*, *hyc* and *hyf* operons (Sawers, 1994; Andrews et al., 1997; Trchounian et al., 2012). They catalyse the reversible reaction [Eqn 19]. These H_{yd} enzymes activities are influenced by various organic acid transporters like succinate and formate (Karapetyan et al., 2020; Babayan et al., 2024), pH, oxygen (Rossman et al., 1991; Alavi-Borazjani et al., 2024) etc.



3. Challenges and limitations in bio-hythane production

Waste-derived bio-hydrogen and the other energy forms discussed in this current context offer great scope in attaining practicality at a larger scale by reconsidering fewer factors such as policies and regulations that would eventually favour the emergence of a circular green economy. Biohythane is emerged as a future potential transportation fuel and its production from organic waste is having major challenges and limitations and the crucial factors are listed below: (i) nature

of biocatalysts, (ii) mixed culture, (iii) operational parameters (pH & temperature), (iv) metabolic limitations and (v) storage/dispensing stations.

3.1. Nature of Biocatalyst

Table 1 summarizes the major bio-hythane producing biocatalyst. Bio-H₂ producing catalyst broadly classified into a group of facultative anaerobes and strict anaerobes which includes pure culture, mixed enriched culture, co-cultures. Besides syntrophic microbial metabolism also co-exists within the anaerobic hydrogen producing microbial community. The syntrophic microbial community are sub-dominant microbiota, which plays a vital role in stabilizing the hydrogen producing microbial community. For instance, Hung et al., (Hung et al., 2011) demonstrates that the presence of *Streptococcus* sp. along with *Clostridium* sp. facilitates the granular formation in the high-rate hydrogen producing reactors. Additionally, various lactate-driven bacteria such as *Sporolactobacillus* sp. also co-exist in the high-rate hydrogen producing reactors (Kumar et al., 2016; Sivagurunathan et al., 2016a).

Table1

Recently Park et al., (2021) conclude that co-culture of lactic acid producing bacteria, *Sporolactobacillus vineae* and well known H₂ producing bacteria *Clostridium butyricum* stimulates the bio-H₂ production. The enhanced H₂ formation is observed with significant increase in the formation of acetate and butyrate. Additionally, the gene expression data implies that *C. butyricum* converted the lactate into butyrate and H₂ via additional cross-feeding metabolic bio-stimulator pathway. Besides co-cultures, monocultures of facultative anaerobic bacteria such as *E. coli* is applied to utilize various organic wastes of agro-industrial origin to produce effectively bio-H₂ which can be further used in two-stage fermentation process for bio-CH₄ or biohythane production. Particularly, *E. coli* has been employed to utilize the wastes of beer, alcohol, coffee, and dairy industries (Poladyan et al., 2018; Bekbayev et al., 2024) or mixture of various agro-industrial wastes accompanied with glycerol (generated during bio-diesel production) (Mirzoyan et al., 2020).

3.2. Enriched mixed culture (EMC)

The EMC play a vital role in two-stage hythane production as it provides better process efficiency with wide array of substrate utilization and a suitable alternative to the existing pure/co-culture system. Additionally, the enriched mixed consortia are having better tolerance to external factors such as pH, temperature, organic loading rate with minimal media requirement for their

cultivation. The EMC addition stimulate the H₂ production at acidogenic-phase *via* enhancement of hydrolysis and metabolism of hydrolysis products *via* active functional consortium with diverse metabolic pathway (Sivagurunathan et al., 2014). The H₂ producers exist in natural environment along with other H₂ consumers such as methanogens, homoacetogens, EMC can be obtained from soil, sludge, wastewater treatment plants, compost, *etc.* with appropriate pretreatment techniques to suppress the activity of hydrogen consumers. As recently overviewed by Pachapur et al., (2019) thermal treatment is widely practised methodology to enrich the active-hydrogen producers, followed by chemical agents, other physico-chemical or combination methods and also reported to develop an EMC. The active role of thermal treatment strategy for EMC development is facilitate the stability under harsh conditions and spore forming capacity. However, the major short coming with this method is other useful non-spore forming hydrogen producers gets vanished from the microbial community dynamics. Alternative to thermal pretreatment various treatment methods such as chemical, aeration, repeated-batch cultivation, physical or combination pretreatment provided a significant improvement in activity of hydrogen production *via* selection of EMC from the mixed consortia. It is also documented that selection of appropriate pretreatment method for hydrogen production in first-stage is beneficial for enhancement of biohythane production in two-stage system (Sarkar et al., 2021b; Sarkar and Venkata Mohan, 2017a).

4. Influential parameters for bio-hythane production

4.1. pH

In biological process, pH plays an important role as it governs the overall metabolic process *via* managing the redox/flux. Biohythane process *via* two-stage system is pH specific and having different optimal pH conditions. Hydrogen production process is favoured at acidic pH environment with a suitable range of 4.5 to 6.5, whereas methanogenic process prefers alkaline pH range over 7.0. During first-stage of fermentation, the key role is to produce hydrogen along with useful volatile fatty acids (VFA) such as acetic and butyric acid, which is beneficial for methane generation. For instance, the methane yield obtained from acetic acid is about 1 mol CH₄ / mol of acetic acid, whereas from butyrate the yield is slightly higher 2.5 mol CH₄ / mol butyric acid. The production of other VFA such as propionic acid limits the hydrogen yield and methane production in the latter stage of the fermentation. Patel et al., (Patel et al., 2015) also demonstrated that hydrogen and VFA production is pH specific. The isolated hydrogen producing bacteria

Clostridium sp. IODB-O3 produces maximum hydrogen yield of 2.61 mol-H₂/mol-sugar VFA generation dominated with butyric and acetic acid at initial alkaline pH of 8.5. Sarkar et al., (2022, 2021b, 2021a)), studied the effect of initial uncontrolled pH in the range of (4–10) for H₂ and VFAs production from brewery spent grains. The study demonstrated that initial pH significantly influences the hydrogen and VFAs production, maximum of about 833 mL and 10.2 g/L, respectively, were noticed under initial alkaline pH of 9.0. In another report Sarkar et al., (2021b) demonstrated that initial pH operation during first-stage of fermentation is crucial for enhanced bio-hythane production from FW. It has been demonstrated that it is possible to produce H₂ at very acidic pH (pH 2.7) (Mota et al., 2018). The bio-hythane production with pH 6 and 7 operation at first-stage and pH 7 at second-stage provided a hythane yield of 3.57 and 4.25 L in an integrated process. Regulating the appropriate pH is important to redirect the metabolic flux towards for enhanced bio-H₂ production followed by successful bio-CH₄ production in the later stage.

4.2. Temperature

Temperature is another critical factor influencing the potential of bio-hythane production as it influences the substrate hydrolysis, metabolic flow, cell biomass growth and end-products formation. The optimal temperature ranges for mesophilic (25–45 °C) and thermophilic (50–55 °C) is ideal for biogas production as the biochemical reactions are favoured with temperature phase changes (Shanmugam et al., 2021b; Xia et al., 2016). Chen et al., (Chen et al., 2021), reported temperature phase assisted [mesophilic (H₂) –thermophilic (CH₄), (thermophilic (H₂)–mesophilic (CH₄)] two-stage biohythane production from co-digested rice straw and pig manure. The study demonstrated that thermophilic conditions at the first-stage (H₂) followed by mesophilic temperature at second-stage (CH₄) favour the hythane production from co-digestion substrate. A maximum gas yield of hydrogen (16 mL/gVS) and methane (197 mL/gVS) obtained from thermophilic acidogenic fermentation, followed by mesophilic AD process was significantly higher than the mesophilic–thermophilic (3 mL H₂/ gVS and 109 mL CH₄/g VS) conditions in two-stage hythane production. This was due to the better hydrolysis of the biomass under thermophilic conditions, which triggered the hydrogen and VFAs generation at first-stage, followed by subsequent conversion of VFA to methane with high yield. The microbial analysis showed that the temperature assisted two-stage hythane production greatly influenced by the success of the first-stage hydrogen producing acidogenic reactor. In the mesophilic conditions, the low yield of hydrogen production is related to the dominance of hydrogen consumer

Methanobrevibacter i.e., over 75% of the total microbial population, which is a hydrogenotrophic methanogen. Whereas the initial operation at thermophilic conditions revealed the abundance of hydrogen-producing bacteria over 50%, which has resulted in enhanced hydrogen and VFAs generation that further transformed into methane in a mesophilic reactor. Thereby, Gadow et al., (2024) verified that temperature plays a significant role in enhancing the generation of bio-hythane.

4.3. Metabolic limitations

The study on biohythane producers, *i.e.*, H₂ producing bacteria, and CH₄-forming archaea and their metabolic interactions will provide useful insights about the feasibility of the bio-hythane production from organic waste. Each specific gas producers involved multi-step / enzyme reactions to produce the desired biohythane gas. For instance, in hydrogen production metabolic pathway, the major limitation is the formation of other by-products such as lactic acid, ethanol, propionate, which hinders the hydrogen formation *via* diverting the reducing equivalents *i.e.*, NAD⁺/NADH and H⁺. In mixed-type fermentation predominant with acetate and butyrate metabolism the H₂ yield was usually observed $\sim < 2.5$ mol H₂ / mol hexose (Cremonez et al., 2021; Rajesh Banu et al., 2021; Sarkar et al., 2021b). The other competing pathway such as homoacetogenesis, propionic acid and lactic acid formation also co-exist within the DF process due to the diverse nature of the biocatalyst involved. The operation of DF reactor with pure culture could provide selective hydrogen production ability without the major shift in the metabolic pathway but DF operation with pure culture is inappropriate for large-scale applications owing to the higher operational and maintenance cost (Sivagurunathan et al., 2016b). For example, Sivagurunathan et al. (2014) demonstrated that during continuous hydrogen fermentation using beverage wastewater, the presence of propionic acid-producing bacteria shifted the metabolic profile in the hydrogen-producing reactor, resulting in a propionic acid concentration exceeding 2.3 g/L, which adversely impacted hydrogen production rates and yield (Sivagurunathan et al., 2014). Applying an intermittent temperature-shift strategy successfully reduced the activity of these bacteria, improving hydrogen production performance by over 60%.

In another study, Sivagurunathan et al., (2017) observed that a repeated heat treatment strategy (80 °C for 30 minutes) in a H₂-producing UASB reactor fed with galactose enhanced hydrogen production by 27%, driven by shifts in metabolic profiles and microbial community

dynamics (Sivagurunathan et al., 2017). Prior to the temperature shift, the reactor was dominated by *Proteobacteria* (61.3%), *Bacteroidetes* (24%), and *Firmicutes* (14.5%), producing lactic and propionic acids alongside acetate and butyrate. Post-temperature shift in the microbial population showed that *Firmicutes* has increased to 88% while *Proteobacteria* dropped below 11%, leading to reduced lactic and propionic acid production and increased acetic and butyric acid levels, which boosted H₂-production efficiency. However, Sivagurunathan et al. (2016c) noted that enriched mixed cultures from repeated batch operations may not always be suitable for continuous processes (Sivagurunathan et al., 2016c). Changes in microbial community composition, particularly increased *Sporolactobacillus* sp. and reduced *Clostridium* sp., caused shifts in metabolic products, significantly affecting H₂-production efficiency. Kim et al. (2008) further reported that early transition from batch to continuous mode can suppress lactic and propionic acid bacteria activity (Kim et al., 2008). An additional challenge in DF is the co-existence of homoacetogenesis, where CO₂ and H₂ are converted reductively into acetate. Saady (2013) extensively reviewed this process in DF, proposing that effective CO₂ removal is essential to mitigate homoacetogenesis and enhance hydrogen yield (Saady, 2013).

Another promising approach to promote DF and metabolic limitations is bio-augmentation. In bio-augmentation, the selective addition of either mono or co-culture or a mixed flora to a native microbial population to stimulate the hydrogen production *via* metabolic pathway redirection and also provide wider substrate utilization ability due to the broader catabolic enzymes involved. Kumar et al., (2015) demonstrated that potential bio-augmentation of facultative anaerobic bacteria (*E. coli*) with mixed consortia (enriched with *Clostridium* sp.) along with supplementation of nitrogen sources (tryptone & yeast extract) stimulated the hydrogen production from industrial wastewater. Kotay and Das, (2010) demonstrated that defined consortium comprises of *Enterobacter cloacae*, *Citrobacter freundii* and *Bacillus coagulans* stimulated the hydrogen production over 11% from sewage sludge than the mono-culture of the individual strains tested. Ren et al., (Ren et al., 2008) potentially tested the role of bio-augmentation on hydrogen production *via* pooling different hydrogen producing bacterial sp. augmentation of *Clostridium acetobutylicum* with *Ethanoigenens harbinense* improved hydrogen production from CMC with a shift in metabolic profile dominant with acetate, butyrate and ethanol as products (Pandey et al., 2023). Goud et al., (2014) reported that augmenting acidogenic microflora with *Bacillus subtilis* improved the bio-hydrogen production *via* redirecting the metabolic flux towards acetate, butyrate

with no formation of propionic at higher organic loading rates. In general, the successful role of augmentation on DF is relied on (i) adaption of augmented catalyst into the existing system, (ii) better substrate degradation ability, and (iii) enhancing the process performances. In AD process, the major metabolic limitation is the interspecies H_2 transfer, hydrogen partial pressure and direct electron transfer and it can be improved *via* addition of bio-stimulators such as granular activated carbon, metallic nano-particles or *via* integration of MES system to stimulate the IHT and DIET (Anukam et al., 2019). Further, the accumulation of VFAs also leads to metabolic in-balance in the CH_4 -producing pathway. The hyper accumulation of propionic and butyric acid in the first-stage process need to be controlled below 8 g/L as they possess serious metabolic hindrance to the AD process in second-stage of fermentation (Mamimin et al., 2017).

4.4. Reactor design

Bioreactor design plays a crucial role in biohythane production from organic waste as it governs the growth and activity of the desired microbial populations to recover energy recovery in the form of H_2 and CH_4 from the complex organic feedstock. Compared to stand alone single stage AD process for biogas production, biohythane production is achieved *via* two-stage or multistage reactor, in which enhanced energy recovery is attained from the organic waste due to improved substrate hydrolysis at first-stage with formation of H_2 and VFAs and subsequent transformation of acidogenic VFA effluent to methane gas. However, the cost of biohythane process is slightly higher than single-stage AD process owing to the additional reactor requirements, heating exchange system to maintain the desired temperature for each process, recirculation, and transfer of effluents from acidogenic reactor to methanogenic reactor (Rajendran et al., 2020).

Another challenge in biohythane production is successful integration of two-stage process. The first-phase *i.e.*, acidogenic phase is crucial as it provide H_2 and VFA formation, the formed VFAs is further transformed into CH_4 in the second reactor. Hydrogenotrophic methanogens or other H_2 competing microbes exist in the acidogenic mixed culture. Appropriate inoculum conditioning such as heat-shock or chemical method is required to enrich the H_2 producers, and the enriched H_2 producers provided the stable hydrogen production. Further, the over-acidification in the second-stage reactor might upset the metabolic process in the AD, or washout of the methanogens at lower HRT (Guneratnam et al., 2017). Additionally, the reaction kinetics of two-stage fermentation is pH specific. The first-stage is conducted within pH range of 5.5–6.5, whereas

the second-stage is operated at pH 7.0–8.0. In large-scale reactor maintenance of pH is bit challenging due to huge requirement of chemicals and earlier attempts were tried to maintain desired acidic pH in the reactor *via* recirculation of the methanogenic effluent back into the first-stage process as it provided the better neutralization ability with low chemical and water requirement. Table 2 summarizes the types of various complex organic wastes using batch reactors. In continuous mode of operation CSTR reactor is widely used for solid organic residues due to better handling of higher solids content over 8–12% of TS, another predominantly studied reactor for biohythane production is UASB reactor, which is commonly used for the application of low–high strength industrial wastewater feedstock. Additionally, the SDSAR is used for handling higher organic loading rate, as it provides the improvement in the mixing ability *via* siphon flow. The SDSAR is alternative to the commonly used continuous mixing/stirring reactor, in which, the operation improves the digestion performance, reduced accumulation of VFA, and scum layer formation (Kobayashi et al., 2013).

Pasupuleti and Mohan (2015), demonstrated a semi-pilot scale hybrid reactor for biohythane production in a single-stage fermentation using spent-wash as a substrate. Under fed-batch mode operation of successive five cycles of operation the cumulative biohythane production was noted as 147 L with COD removal efficiency of over 66% and the biogas enriched with bio-H₂ (23.5 %) and bio-CH₄ (76.5%), respectively. Ta et al., (2020) demonstrated that single-stage novel CSTR reactor is used for the production of biohythane using entrapped microorganisms. The bio-H₂ and bio-CH₄ producing microbes were entrapped in the form of immobilized cells and they were used for biohythane production under various HRT from industrial wastewater. This is the first report making use of single-stage system for the production of biohythane using immobilized cells as a biocatalyst. In another report, Thi Nguyen et al., (2021) demonstrated that maintaining optimum food to microbe ratio is essential for biohythane production in a single-stage process. The authors demonstrated the biohythane production ability of food waste and molasses wastewater in a single-stage system under batch mode operation and the composition of H₂ and CH₄ depends on the tested F/M ratio.

Recently, Si et al., (2020) demonstrated an integrated multistage biohythane reactor and compare the performance with stand-alone UASB reactor. The results showed that novel reactor design provide better biohythane production ability in comparison to UASB and the hydrogen content in the biohythane gas was dependent on the applied HRT. The microbial community

analysis revealed that the modified reactor harbouring the bacterial community rich in acidogenic, acetogenic, and methanogenic archaea populations, provided an efficient syntrophic mechanism for continuous biohydrogen production. Gadow and Li., (2020) demonstrated the operational stability of the single-stage biohydrogen production in a CSTR system and using cellulose as a substrate during long term operation over 240 days. Operational of the reactor with a stable pH range of 6.0–6.5 is feasible condition for single-stage biohydrogen production from cellulose rich wastewater and it provided a treatment efficiency over 65%.

4.5. Bio-stimulators

Anaerobic microbes require certain metal co-factor/stimulators to regulate their metabolism for cell growth and product formation. In recent year, various bio-stimulators such as nanoparticles, trace elements, granular activated carbon, fly ash are reported to enhance the productivity of H_2 and CH_4 . The intrinsic role of bio-stimulators is that it regulates the metabolism towards selective production of targeted gaseous products. Mamimin et al., (2019b) reported that addition of trace metals such as Ni/Co/Fe at a concentration of 10, 6, and 20 mg/L promoted the two-stage biohydrogen production from POME (palm oil mill effluent). Trace metals addition enrich the hydrogen producers such as *Bacillus* sp, *Clostridium* sp., and *Thermoanaerobacterium* sp., which promotes the hydrogenase gene expression level. Further the second-stage fermentation also accompanied with increasing abundance of *Methanosarcina*, *Methanomassiliicoccus*, and *Methanoculleus*, which led to improvement in biogas yield over 21% and COD removal efficiency of over 95%. Lu et al., (2017) demonstrated that addition of Fe_3O_4 - zeolite promotes the acidogenesis/hydrolysis and VFA synthesis dominant with acetate formation in the first-stage from complex substrate rice straw and cow manure. The resultant digestate stimulates the methane production in the second-stage with an increment in methane yield over 78% than the control reactor with no addition of nanomaterials. Zhang et al., (2020) overviewed that Fe_3O_4 nanoparticles stimulated the biohydrogen production from waste activated sludge. Addition of 100 mg/L of iron oxide nanoparticles yielded 11 mL H_2 /g Vs and 109 mL CH_4 /g Vs, which is over 15% and 58% higher gas yield than the control reactor. Fe_3O_4 nanoparticles promote the sludge disintegration with an increased release of soluble COD and carbohydrates fraction available in the sludge. Park et al., (2019) demonstrated that addition of granular activated carbon enhances the bio- H_2 production in *Clostridium butyricum* via altering the metabolic flux towards the effective formation of acetate and butyrate with lowering the formation of lactate. The functional

transcriptome gene analysis revealed that functional genes responsible for hydrogen production gets stimulated with a reduction in gene expression level of hydrogen consuming pathways *via ldh* enzymatic system. The careful selection and addition of bio-stimulators could promote the efficiency of two-stage biohythane production.

4.6. Co-digestion

Co-digestion is a process of utilization of different mixture of homogenous waste to achieve higher biohythane yields. Co-digestion provide better synergy, enhanced nutrient supplement, optimal carbon to nitrogen ratio, and better buffering capacity to improve the biohythane production. Jehlee et al., (2019) demonstrated that co-digestion of algal biomass with organic waste enhanced the biohythane production in a two-stage AD process. The optimal C/N ratio varied between (25 – 30 %) co-substrates used. Co-digestion of algal biomass with organic waste provided synergic effect on enhancing bio-H₂ and bio-CH₄ than the mono-digestion. Co-digestion of algal biomass with glycerol, palm oil mill effluent, molasses, nappier grass, palm oil empty fruit bunch) gave overall biohythane yield of 409 to 616 L/Kg biomass with an optimal H₂ ratio of 6.3 to 22.0 % in the hythane mixture. Mono-digestion of algal biomass gave biohythane yield of 202 L/ Kg biomass with a H₂ ration of 17.8. Mishra et al., (2017) reported that co-digestion of distillery effluent with de-oiled ground cake enhanced the biohythane production as it provides the nutrient balance to the process. About 2.5-fold increment in biohythane production with a maximum gaseous energy recovery of 23 kcal/L. Mamimin et al., (Mamimin et al., 2019a) observed that co-digestion of palm oil mill effluent with solid wastes generated in the palm industry improved the biohythane yield over 113%, when compared with the POME alone. Co-digestion of the liquid waste with solid organic wastes provides less energy demanding hydrolysis, to increase the degradation rate of organic contents with dilution process of toxic ingredients present in the wastes that provides synergistic effects on enhancement on biohythane production. Few reports also demonstrated the performance of co-digestion in continuous mode operation. For instance, Kumari and Das, (Kumari and Das, 2019) reported that co-digestion of alkali/ enzymatic pretreated sugarcane baggase slurry with water hyacinth promoted the biohythane production at an optimal HRT of 8 h and 10 d for two-stage biohythane production. The study provided the biohythane yield of 119 L/Kg COD with a H₂ content of 22%. Qin et al., (2019), demonstrated that biohythane production from co-digestion of FW and paper waste in a recirculated two-phase AD system and achieved H₂ and CH₄ yields of 78 L/Kg-Vs and 373 L/Kg Vs with an increment

in higher heating value over 13% than the food waste alone. Chen et al., (Chen et al., 2021) reported two-stage temperature phase AD process for biohythane production from rice straw and swine manure and observed a relatively higher biohythane production from thermophilic- H_2 and mesophilic- CH_4 than mesophilic- H_2 and thermophilic- CH_4 operation. Thermophilic operation at first-stage promotes the efficient hydrolysis of the co-substrate with an abundance of hydrogen-producers (*Thermoanaerobacter* and *Clostridium* sp) and selective pressure to suppress hydrogen consumers, which resulted in efficient biohythane production in a two-stage operation (Yield = 213 L/Kg Vs). The mesophilic-operation at first-stage observed the presence of hydrogenotrophic methanogens and attained low bio- H_2 yield of 3 mL/gVs, followed by a low CH_4 yield of 109 mL/gVs. For efficient two-stage biohythane production, bio- H_2 production in the first-stage is crucial for the overall performance of AD.

4.6.1. Pretreatment

Pretreatment is an important aspect in two-stage AD process. In acidogenic phase, inoculum was pretreated to suppress the activity of hydrogen consumers in the mixed microbial population dynamics. Commonly for the acidogenic inoculum various physico-chemical pretreatment method such as heat-shock, acid, alkali, and chemicals were used. A combination of pretreatment method also used to activate the spore-forming hydrogen producers such as *Clostridium* sp, *Bacillus* sp. The pretreatment step is required for the removal of lignin, reducing the complexity of the biomass structure, increasing the accessibility to microbes-substrate interaction and promotes the substrate degradation for efficient biogas production. Various methods such as hydrothermal, ultrasonication, acid, alkali, and steam-explosion were tested and proven to improve the better biogas production ability from organic wastes. Jafari and Zilouei., (2016) reported that sugar cane bagasse pretreated with a combination of TiO_2 , ultraviolet irradiation and dilute sulfuric acid improved the substrate hydrolysis and resulted in more enhancement in bio- H_2 and bio- CH_4 production over 127 and 57%, respectively than the untreated substrate. Baeta et al., (2016) reported a biohythane production from hemicellulose hydrolysis fraction of sugarcane bagasse obtained *via* autohydrolysis pretreatment. The acidogenic step was promoted to reduce the toxicity of the hemicellulose hydrolysate and resulted in 4-fold enhanced methane production in the second-stage. Jehlee et al., (2019; 2017) obtained a bio- H_2 and bio- CH_4 yields of 190 and 319 mL/g Vs, respectively from hydrothermally pretreated algal biomass (*Chlorella* sp). It is about one-fold higher than that of the untreated biomass. Kumari and Das

(2019) utilized biologically pretreated sugarcane tops, using a fungal strain (21 days) for biohythane production and showed that such a pretreatment is feasible for removing ~60% of lignin with a cumulative biohythane yield of 235 L hythane / Kg biomass with a gaseous energy recovery of 37%. In another report, Kumari and Das (2019) showed that alkaline peroxide pretreated sugar cane baggase (50 °C for 150 min) provided lignin removal over 89%, and the resultant solid residue was subjected to enzymatic hydrolysis and used as a co-substrate along with water hyacinth and provided a yield of 119 L biohythane /Kg biomass.

4.7. Storage/dispensing stations

Biohythane is a viable future fuel and a step towards promoting the hydrogen economy in the existing fuel system. It is having a typical hydrogen content of 8 to 20% in methane gas provided better combustion and reduction in air pollutants such as NO_x and CO₂, and can be used in the existing CNG infra-structure without any significant changes. At present, the hydrogen and methane gas are stored separately in a dedicated storage unit due to the intrinsic properties of both gases. Typically, large scale hydrogen is stored in a underground storage system at higher pressure (350–700 bar) and cryogenic storage at lower temperature (–250 °C), wherein it is suitable for the stationary applications. For mobile applications, H₂ is stored in metal hydrides under moderate pressure. Hassan et al. (2021) provided a detailed review on challenges associated with in H₂ storage using metal hydrides for on-board applications. For instance, 1 kg of H₂ is about 11,200 L, whereas it can be reduced to 26.3 L when compressed at 70 MPa. At liquid hydrogen storage mode this can be further reduced to 14.1 L at temperature of 20 K. Mg(BH₄)₂ like metal hydride can store H₂ in a small volume of 8.2 L. Being used as a transportation fuel, compressed natural gas (CNG) has better storage ability and dispensing stations. Despite, the efforts to replace CNG with HCNG various research organization and government agencies is working on the direction to improve the storage and dispensing station of biohythane as a transportation fuel.

4.8. The effect of interaction between various influential parameters for biohythane production

The volume of bioreactor determines HRT, whereas the bioreactor dominant microbial community affects the SRT. Both are equivalent in conventional low-rate bioreactors that do not recycle or remove supernatant. However, SRT is significantly higher than HRT in high-rate bioreactors (Alavi-Borazjani et al., 2024). The HRT values for the first hydrogenogenic stage

should be between 3 and 10 days, according to the literature (Wirasembada et al. 2024), and for the second methanogenesis stage, the period could be between 3.5 and 25 days (Tsigkou et al. 2023). Depending on the influential parameters for biohythane production, such as feedstock properties, retention time, and operating temperature, the AD systems in conventional single-stage process possess ideal OLR that normally ranges from 0.5 to 2 kg Vs / m³ / day (Ramanathan et al. 2022). On the other hand, two-stage AD systems isolate the methanogenesis and fermentation processes for different microbial communities. The study found that these systems can handle organic loads up to 300% higher than single-stage AD systems (Rajendran et al. 2020). Further, high nutritional nitrogen concentrations in bioreactors can interfere with microbial activity and have a detrimental effect on process stability and efficiency because of the presence of NH₃. It changes the intracellular pH and proton imbalance. On the other hand, PO₄³⁻ nutrient supplies the building blocks for ATPs and nucleic acids and aids in preserving the bacterial functions throughout the fermentation. A higher phosphate content during DF results in more bio-H₂ production (Thong et al. 2018). The findings showed that the rate of biohythane is increased when short HRTs are allocated to the production of bio-H₂ while longer HRTs are assigned to bio-CH₄ production. Consequently, the food processing wastes yield a high bio-CH₄ yield (0.49 m³ kg⁻¹ VS during a 7 days HRT. The shorter HRT will result in a high OLR, which causes VFAs, ultimately lower the pH to limit methanogenic activity. Further, it has showed that *Methanobacterium beijingense* and *Methanothrix soehngenii* produced more bio-CH₄ yield in a hydrogenogenic reactor, while *Clostridium butyricum* produced more bio-H₂ yield (Meenu Hans et al., 2019). Similarly, it was found that *Clostridiaceae* / *Ruminococcaceae* and *Enterobacteriaceae* strains from food waste yielded bio-H₂ production of about 101.75 ± 3.71 L H₂ kg⁻¹, an OLR of 126.67 kg COD m³ d, and 3.37 kg COD m³ d that were obtained by combining OMW with cheese whey to produce more bio-H₂ and bio-CH₄ yield (Ramakrishnan Anu Alias Meena et al., 2020). Similarly, two distinct OLRs were used to treat the sugary wastewater from the sugar refining industry. The highest rates of bio-H₂ and bio-CH₄ production occurred at an OLR of 2.1 g COD L⁻¹ d⁻¹ and 6 g L⁻¹, respectively (Jaya Prasanna Kumar et al., 2024). The effects of OLR, specifically on the simultaneous synthesis of biohythane in a two-stage AD system, have not yet been thoroughly examined in research. Understanding the effects of OLR on the microbial community and its properties has been the main focus of recent research. One study, for example, showed a strong relationship between the microorganisms and the genes of AHLs in the recovery

of UASB. Furthermore, impact of OLR on methane production in UASB has been the main focus of earlier research. Other recent studies have shown that, under the impact of a species within the genus *Anaerovorax*, OLR can interfere with the complex microbial interaction necessary to maintain a stable AD. Furthermore, a two-stage AD system with various OLRs was used to assess biohythane production, which increased steadily from 25 to 125 kg COD/m³. Xiaona An et al., (2024). The findings demonstrated that the OLR 75 kg COD/m³ produced the highest biohythane. Furthermore, the process failure and post-failure restoration following a OLR were not examined in earlier research. The bio-CH₄ production rate (BMPR), hydrogen content, and bio-H₂ yield peaked at 1.003 L CH₄/L·d, 1.641 mol H₂/mol glucose, and 62.61%, respectively, during the OLR phase before sharply dropping. Interestingly, during the restorative phase, the bio-hydrogen production sharply declined to nearly zero, while bio-CH₄ production exhibited a resilience and reached its peak methane content of 52.2 % (Velusamy Mozhiarasi et al., 2023).

It is known that the ideal C/N ratio for the AD process is in between 20 and 30 (Zhang et al. 2014). By raising VFAs as well as elemental P levels especially at 3.3 mg/L, and lowering the pH, can have a detrimental effect on methanogenesis (Thong et al. 2018). Furthermore, a feedstock carbon / nitrogen (C/N) ratio should be higher than 20 for steady bio-H₂ fermentation. However, all these possess to have less effect on acidogenesis (John Ngabala and Kamuhabwa Emmanuel 2024). When the bio-H₂ fermentative bacteria were cultivated in fermentation media with a C/N ratio higher than 20, their bio-H₂ production can potentially be improved. The stage of bio-CH₄ production is positively impacted by a C/N ratio of 20–30 (Thong et al. 2018). Some studies suggest that 3000 mg/L of SO₄²⁻ nutrient at pH 5.5 may be advantageous for bio-H₂ production, despite the fact that this nutrient concentration influences DF (Jaya Prasanna Kumar et al., 2024; Mohamed Abbas Soltan et al., 2024). Although, the levels between 100 and 800 mg/L are inhospitable to methanogens, high dissolved S²⁻ nutrient concentrations (> 100 mg/L) can stop the production of bio-H₂ during the DF process. According to Chandrasekhar et al., (2015) each micronutrient plays a unique role in microbial metabolism, and variations in its bioavailability can obstruct this process. Thus, it is essential to carefully control these micronutrient levels in order to encourage the best possible microbial growth and prevent any negative impacts. Additionally, for the DF, the inoculum size was adjusted in between 10 and 20% (v/v). The thermophilic fermentation may be improved by the batch reactor with nitrogen to lower the HPR and this process with *Caldicellulosiruptor saccharolyticus* will result in a high HPR as well as the oxidation of

NADH electron carrier of the cell, primarily to lactate (Christian Peintner et al., 2010). Thereby, a high HPR may be the source of lactate production under unstable or overloaded situations. A steady production rate of bio-CH₄ is defined by the bicarbonate alkalinity, or CaCO₃, in the 1000–5000 mg/L range. The ideal range for the VFA to alkalinity ratio is in between 0.15 and 0.25. A higher temperature causes the retention time process to go considerably more quickly, which leads to more efficient operation and a shorter retention time requirement (Thong et al. 2018). The micronutrients such as Na⁺, Mg²⁺, Fe²⁺, Ni²⁺, Co²⁺ and Zn²⁺ are essential for the production of biohydrothane. For DF, such nutrients must be added to the media to facilitate the transport pathways, and building of structural skeletons for numerous enzymes, including Fe-Fe hydrogenase and Ni-Fe hydrogenase (Chinchusak et al. 2023). In the bio-CH₄ production step, the Fe²⁺ nutrient is very essential in order to generate protocheme *via* precorrin-2, which is created from uroporphyrinogen III protein in two sequential methylation reactions using S-adenosyl-L-methionine in the presence of methanogenic archaea like *Methanosarcina barkeri* (O-Thong et al., 2018). In order to achieve acceptable performance, the methanogenic reactor is primarily optimized by adjusting OLRs either raising the substrate concentration or lowering the HRTs (Tasneem Abbasi and Abbasi 2012). A drop in pH is one of the primary indicators of methanogenic reactor overloading or instability. The VFA accumulation is actually influenced by a decrease in pH, thereby the biohydrothane participating in the second-stage of AD process are affected by pH levels below 6.3 in terms of the enzymatic activities. So, it is believed that a pH range of 6.5 to 7.8 is suitable for methanogenic archaea (Mohanakrishnan Logan et al. 2023).

5. Futuristic approaches in molecular tools/genetic tools

The advancement in the molecular/genetic tools pave a way for the gene modification and redirect the redox balance of the bacterial metabolism. H₂-production pathway involves mixed-acid type fermentation, in which reducing equivalents (NAD⁺ / NADH) gets diverted to the formation of various mixed-acids. For efficient hydrogen production using metabolic engineering approach the targeted pathway modification requires careful selection to redirect the metabolic sink towards hydrogen production. Li et al., (Li et al., 2020; 2019) engineered the thermophilic bacteria *Thermoanaerobacterium* sp. *via* knockout the NADH-dependent reduced ferredoxin: NADP⁺ oxidoreductase (NfnAB) pathway for the improvement of hydrogen and ethanol production with a reduction in lactate formation. The engineered strain was tested for various acid-hydrolyzed biomasses and provided the hydrogen yield of 1.66–1.77 mol/mol substrate. In another

study, Li et al., (Li et al., 2010) modified the *Thermoanaerobacterium* with a deletion of L-lactate dehydrogenase gene (ldh), responsible for lactic acid production and the resultant mutant provided a higher hydrogen yield from glucose with a value of 2.8 mol/mol hexose. However, when the mutant tested with glucose/xylose mixture the hydrogen yield was reduced to 2.2 mol/mol substrate. Ito et al., (Ito et al., 2004) modified the *Enterobacter* sp by exposing the bacterial cells to chemical mutagenesis with NTG and the resultant mutant formed less amount of lactate and 2,3-butanediol with an enhancement in hydrogen yield 1.8 mol/mol glucose, which is higher than the negative control value of 0.8 mol/mol hexose. Similarly, Roy et al., (2015) used chemical mutagen to alter the bacterial cell metabolism. *Thermoanaerobacterium* sp. cells were exposed to ethyl methane sulfonate and the mutant cell redirects $\text{NAD}^+ / \text{NADH}$ towards the formation of more amount of hydrogen with less alcohol formation. The resultant mutant provided the hydrogen yield of 3.12 mol/mol hexose. The mutant cells were also evaluated for hydrogen production from starch wastewater in first-stage followed by methane generation in the second-stage reactor provide overall COD removal efficiency of 66% with gaseous energy recovery of 53%.

6. Application of MEC system for biohythane

MECs are well-established alternative methods for converting carbon-rich materials into high-value products like bio- H_2 and bio- CH_4 (Chaurasia and Mondal, 2022; Rikame et al., 2018). Various carbon-rich substrates, including waste or wastewater from distilleries, swine farms, sewage, food and dairy industries, and LCB have been effectively utilized for bio- H_2 and bio- CH_4 production through MECs (Kadier et al., 2014). MECs operate in both continuous and batch modes, yielding substantial biohythane production (Tartakovsky et al., 2009). However, certain factors can impact MEC efficiency, influencing both bio- H_2 and bio- CH_4 yields and substrate degradation rates (Pandey et al., 2022; Sarkar et al., 2023b). In MEC systems, the EMs act as biocatalysts, facilitating electrochemical reactions at the electrode interface. Through a series of enzyme- or microbe-mediated steps, these EMs contribute to higher biohythane yields by catalysing reactions that enhance production efficiency. In the electro-methanogenesis process for bio- CH_4 production, the EMs transfer electrons to the electrode surface either directly or *via* electron shuttles and mediators. The biohythane yield in this process largely depends on the choice and pretreatment of EMs or biocatalysts. Pretreatment can suppress methanogenic bacteria, promoting the growth of other microbes to optimize bio- H_2 production. Beyond the EMs or

biocatalysts, factors such as electrode materials, pH, membrane type, temperature, applied voltage, and system architecture are key determinants of MEC performance.

In MEC systems, both anode and cathode materials are essential, as they facilitate biofilm formation, create active redox catalytic areas, and provide electroactive interfaces for redox reactions. Ideal electrode materials should be low-cost, eco-friendly, durable, mechanically robust, highly biocompatible, and exhibit favourable surface morphology to enhance product yield and process efficiency (Sarkar et al., 2023b). Additionally, factors like the distance and placement of electrode materials, alongside the chosen materials, significantly impact biohythane yields in MEC systems. The redox reactions can vary based on carbon-rich substrate types and operating conditions such as pH, temperature, and applied voltages. The pH of the medium significantly influences microbial metabolism, impacting the overall yield of biohythane production and the formation of organic acid metabolites. In large-scale biohythane production using MEC systems, key techno-economic factors such as capital costs, scalability, durability, and reproducibility—play a critical role in determining feasibility and performance (Sarkar et al., 2023b).

The electro-methanogenesis of bio-CH₄ production process, the EMs transfer electrons either through direct transfer or shuttles and mediators on to the surface of electrodes. Normally, the production of biohythane yields mainly depends on the choice of EMs or biocatalysts and also the pretreatment of the biocatalyst, which helps to minimize or destroying the methanogenic bacteria thereby, upholding the growth of other microbes' aids to maximum bio-H₂ production. Besides EMs or biocatalysts, other factors like electrode materials, pH, membrane, temperature, applied voltage, system architecture, *etc.*, mainly affect the performance of MEC. In MEC systems, both anode and cathode electrode materials play a vital role *i.e.*, it is assisting the formation of biofilm, which provides an active redox catalytic area, electro-active interface for redox reactions, *etc.* The anode and cathode electrode materials must need to be low cost, eco-friendly, high durable, mechanically strong, high biocompatible as well as significant surface morphology for enhanced product yield and process performance (Sarkar et al., 2023b). Furthermore, the production of biohythane yields in MEC affects the distance between the electrode materials, type of materials chosen and placement of materials in working process. Depending on the type of carbon rich substrates as well as operating conditions such as pH, temperature, applied voltages, *etc.*, initiate the redox reactions. Normally, pH of the medium considerably impacts the bacteria or microbe metabolism leads to affects the entire yield of biohythane production as well as organic

acid metabolites. However, in large scale production of biohythane by MEC system mainly depends on techno-economic aspects such as capital costs, large scalability, durability, and reproducibility (Sarkar et al., 2023b).

7. Techno-economic and environmental assessments

Techno-economic assessment is one of the major steps in commercialising biohythane through an anaerobic digestion process due to its profitability and feasibility. Equipment, installation, engineering, construction, and working capital expenditures for the plant setup and operation until profits are realized and are included in the fixed capital costs of the plant (Vo et al., 2018). The annual cost of producing biohythane includes labour, maintenance and raw material costs, along with laboratory fees. However, the revenue is also generated by solid waste, waste treatment cost, and the commercialization of biogas. Profitability may be evaluated after total expenditures and revenues are determined (Hans and Kumar, 2019). Cost information for various hydrogen generation techniques is available, but nothing is known about the production of bio-H₂ based on DF (Khan et al., 2018b; K. B. Sasidhar et al., 2022). Cost estimation may be aided by the similar production procedures of bio-H₂ and bio-CH₄. Tax cuts and subsidies are examples of government incentives that could lower capital costs and draw investment in the production of biohythane (Mozhiarasi et al., 2023). A two-stage AD plant processing bio-wastes is expected to require an initial expenditure of about 12,687.7 k€, with potential profits of about 54.9 € per ton of bio-wastes. Such projects usually have payback periods that range from two to six years, depending on feedstock composition (Ljunggren and Zacchi, 2010; Micolucci et al., 2018; Mozhiarasi et al., 2023). The profitability of producing biohythane is greatly impacted by the scale of production (Han et al., 2016). By lowering the cost of capital expenditures per unit, economies of scale raise profit margins and lower overall production costs. Larger production volumes, however, call for more feedstock, which could compromise its consistency and quality and increase transportation expenses. Therefore, establishing the ideal production size that strikes a balance between the advantages of economies of scale and the expenses of transportation and feedstock supply is essential for commercial viability. Even after taking into consideration the higher energy requirements for pre-treatment and reactor heating, the two-stage AD process for producing bio-biohythane has a positive energy balance when compared to single-stage approaches (Mozhiarasi et al., 2023). The two-stage process only costs 3% more to set up than a single-stage setup, and the additional energy produced more than makes up for this difference (Rajendran et al., 2020).

All of these investigations point to the two-stage AD process as an economically viable and energy-efficient method of producing biohythane.

Evaluating the economic and environmental effects of biohythane is crucial for its widespread commercialization. AD process for the formation of biohythane has been the subject of few but encouraging environmental investigations. In comparison to single-stage setups, it was found that a two-stage AD system for processing cheese when enhanced energy output and decreased greenhouse gas emissions by 60% (Lembo et al., 2022). Two-stage bio-H₂ and bio-CH₄ generation from wheat had a lower environmental impact than single-stage approaches, and it also had advantages when used as car fuel instead of diesel (Patterson et al., 2013). In a LCE of two-stage biohythane from animal waste, minor environmental impacts were found, with commercial-scale systems exhibiting lower impacts than pilot stages (Sinsuw et al., 2024). Carbon fixation of microalgae reduces the greenhouse gas emissions of 173 g CO₂-eqMJ⁻¹ from the biohythane produced from microalgae and food waste to 124 g CO₂-eqMJ⁻¹ (Chen et al., 2020). Using biohythane derived from maize stalks as car fuel decreased greenhouse emissions more than using it for other purposes. Lastly, the dual-stage AD process produced digestate with reduced levels of acid, elemental N and P, reducing the dangers of acidification and eutrophication. According to techno-economic and environmental research, the production of biohythane by AD is a great promise in sustainable energy and waste management. Capital and operating costs must be balanced for profitability, with government incentives assisting to defray costs. The logistics and quality of feedstock can be difficult; however, the economies of scale help increase the production of biohythane. Particularly, two-stage AD systems surpass conventional techniques in reducing greenhouse gas emissions and environmental effects. These results highlight the significance of thorough assessments to direct the advancement and uptake of biohythane technology, increasing its economic feasibility and environmental advantages.

8. Biohythane integration in waste management systems and Future scope

The current waste management facilities mostly rely on conventional biogas production or landfill disposal, which emphasizes the need for significant infrastructure and technological advancements. Using AD in the production of biohythane has enormous potentiality to enhance the waste management procedures, which offer both financial sustainability and environmental benefits. The creation of biohythane makes it possible to recover valuable materials from waste,

which supports a circular economy. Additionally, compared to traditional biogas production, the concurrent generation of bio-H₂ and bio-CH₄ offers a more efficient alternative by increasing the organic waste-derived total energy output. This involves the utilization of anaerobic digesters that can produce both bio-H₂ and bio-CH₄, the installation of efficient pre-treatment units for the separation and treatment of organic wastes, and the use of sophisticated gas separation and purification systems to maintain the required biohythane composition. This entails implementing efficient pre-treatment units for sorting and treating organic waste, using sophisticated gas separation and purification systems to maintain the desired biohythane composition, and using cutting-edge anaerobic digesters that can produce both bio-H₂ and bio-CH₄.

A comprehensive techno-economic analysis (including initial capital expenditures, continuing operating costs, and market variables influencing methane and hydrogen) is necessary to evaluate the economic viability of biohythane production. Economic viability can also be enhanced by government incentives for waste disposal and renewable energy production. The Government can lessen their reliance on landfills by promoting the expansion of renewable energy, thereby establishing the quality criteria for market acceptance, and passing laws that encourage waste conversion to biohythane using the plants. Additionally, educating the communities about the advantages of biohythane and motivating them to participate in garbage collection, the segregation initiatives can help promote public reception and participation. Working together with non-profits, businesses, and local governments can create a network of support for increasing the production of biohythane. Moving forward, it will be necessary to carry out pilot projects to show viability, make research and development investments to streamline procedures, push for laws that will help, and give professionals the capacity building they need to run biohythane production systems. One way to assess the efficacy of pretreatment procedures is to analyse the concentrations of particular indicators in the dissolved phase of the organic substrate that is undergoing pretreatment. To make this assessment possible, the organic component concentrations are typically tracked. Based on the existence of organic molecules in the dissolved phase, it may be possible in some circumstances to create constant correlations and empirical models to predict AD efficiency and minimize the errors (Archana Kasinath et al., 2021). Practically, while doing such analyses the correlation between the yields of bio-CH₄, & bio-H₂, the concentrations of COD & N-NH₄, and pH & archaea %, are beneficial since it removes the necessity of further performance *i.e.* error analysis. The multiple regression technique has been used to create the empirical formulae

for calculating the production yields of biogas, bio-CH₄, and bio-H₂, because of the linear correlation were taken into account in the calculation. It was found that predictors such the S-CO₂/AGS volume ratio and the COD concentration in the dissolved phase had a statistically significant impact on biogas, bio-CH₄, and bio-H₂ output (Wirasembada et al., 2024).

Since many of the studies used mixed consortia for bio-H₂ production, their community dynamics often vary over a period of time, resulting in insufficient H₂-production. Isolation of microbes and developing a microbial blend with defined culture helps to understand the microbial metabolism and simpler approach for monitoring the community dynamics shift or may improve the performance of microbial culture *via* advance molecular biological tools. The following research directions should be focused in biohythane production process to improve its process efficiency and cost economics. The combined gas from both stages (acidogenic and methanogenic) is rich in H₂ and CH₄, *i.e.*, biohythane and has a high-market value with great potential to replace the existing CNG. Till date, highly biodegradable waste like food waste, molasses, dairy waste, *etc.*, have been studied at pilot-scale. The results address important gaps in the research and establish a standard for renewable energy initiatives in comparable places around the world. They also clearly demonstrate the need of community empowerment and planned interventions in attaining long-term sustainability for biogas systems.

#Table 3

9. Conclusion

The production of biohythane, bio-H₂ in BES systems, the biochemistry of biohythane production, the difficulties and constraints, the influence of the interaction between different influential parameters, futuristic approaches in molecular and genetic tools, the use of MEC systems, the SDG approach to evaluate the scenarios over a ten-year period (2023–2033), techno-economic and environmental assessments, and the integration of biohythane in waste management systems were all covered in this review. Bio-H₂ production from organic waste is hampered by a low conversion yield often, reported to be less than 20%. Hence in order to improve the economics of bio-H₂, a holistic approach *via* various two-stage integration schemes *viz.* bio-CH₄, medium chain fatty acids, alcohols, and bioplastics production should be predicted. The reason of the insufficient gas yields is the remarkable co-formation of soluble metabolites mostly encompassing volatile organic acids and alcohols. This serious hurdle means that a considerable part of the chemical energy stored in the fermentation raw material is diverted to by-product formation and

remains unexploited. DF has been already shown as the most realistic opportunity to leave small (laboratory) scale production behind, which is attributed to the relatively low energy requirements, salient hydrogen production rates and the chance of valorising a broad spectrum of organic matter covering liquid and solid wastes. Herein, we have been concluded the following,

- Screening / selection of a defined bacterial culture with specific hydrogen producers that can be translated into improved stability of the process.
- Developing the defined microbial blend that has ability to withstand high loading rates and acid concentrations is the best solution envisaged in this perspective.
- Constructing the mutants with high H₂-yield, where carbon flux will be redirected towards the H₂ production.
- Applying artificial microbial consortiums for enhanced H₂-production in sole or mixed organic wastes by making use of many microbial species with unique metabolic capacities and combined interactions, results in more dependable and effective bioprocesses.
- Biomass / agriculture residue co-digestion along with this organic waste/wastewater is envisaged to significantly improve the biogas production.
- Low conversion efficiency is also one major challenge, which can be addressed by adapting different strategies, viz., improving the oxygen sensitivity of *hydrogensae* enzyme, addition of cheaper mediators to enhance the H₂-production and also to balance the redox equilibrium, etc. Integration of hybrid electro-assisted system, i.e., microbial electrolysis limits the biocatalyst to enhance the theoretical H₂-production from organic waste / wastewater.
- DF attracts notable attention; it leads up to moderate H₂-yield that is a great problem encountered.
- Additional efforts must be put to recover more amount of energy from the liquid phase residue (fermentation effluent). Another challenge in biological hydrogen fermentation is the instability of the microbial blend.

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Table 1. Challenges in Bio-hythane production.

Challenge	Possible solution
Low biogas/hydrogen yield, production rate and substrate conversion efficiency	Combination of different feedstocks Integration of other processes to utilize the effluents
Sensitive to operational challenges (pH/Temperature/inhibitory agents)	Development of suitable redox balancers
Inoculum instability–(due to the presence of competitive bacteria in the organic waste)	Development of efficient defined microbial blend
Storage and transport of H ₂ to the utilization site	Storage as bio-hythane (blend of H ₂ in CNG). Other storage methods
Higher CAPEX/purification cost	Integration of other processes to valorise other products to balance the economics

Table 2: Bio-hythane production from organic wastes in different scale modes of reactor operations.

Feedstock	Microbial source (H ₂)	Reactor type	Operational time	H ₂ yield (mL H ₂ /g TS)	CH ₄ yield (mL CH ₄ /g TS)	Bio-hythane yield (mL/g TS)	H ₂ ratio (%)	Reference
Steam exploded Corn stalk	Anaerobic sludge	Batch	5 d, 60 d	12	195	207	5.7	(Alavi-Borazjani et al., 2024; Liu et al., 2014)
<i>Sargassum</i> sp.	Anaerobic sludge	Batch	180 h, 42 d	91	541	632	14.4	(Costa et al., 2015)
Wheat bran	Anaerobic sludge	Batch	96 h, 36 d	19	243	262	7.3	(Corneli et al., 2016)
Pretreated Sugar cane top	Anaerobic sludge	Batch	13 h, 20 d	77	157	235	32.9	(Kumari & Das, 2019)
Garden waste	Co-culture	Batch	30 d, 42 d	98	322	420	23.3	(Abreu et al., 2016; Shanmugam et al., 2021a)
Distillery effluent and ground nut deoiled cake	Anaerobic sludge	CSTR–Batch	16 h, 12 d	118#	158#	276	42.8	(Mishra et al., 2017)
Pretreated <i>Chlorella</i> sp	Anaerobic sludge	Batch	5 d, 30 d	190	319	509	37.3	(Abdur Rawoof et al., 2021; Jehlee et al., 2017)
POME + solid residues of Palm oil industry	Anaerobic sludge	Batch	14 d, 50 d	1.6	32.4	34	4.7	(Mamimin et al., 2019)
post-hydrothermal liquefaction wastewater	Anaerobic sludge	Batch	12 d, 21 d	29	254	283	10.2	(Si et al., 2019)
<i>Chlorella</i> sp	Anaerobic sludge	Batch	7 d, 30 d	36	166	202	17.8	(Jehlee et al., 2019)
<i>Chlorella</i> sp and Glycerol	Anaerobic sludge	Batch	7 d, 30 d	39	577	616	6.3	(Jehlee et al., 2019)
<i>Chlorella</i> sp and POME	Anaerobic sludge	Batch	7 d, 30 d	53	545	598	8.9	(Jehlee et al., 2019)
<i>Chlorella</i> sp and molasses	Anaerobic sludge	Batch	7 d, 30 d	90	319	409	22	(Jehlee et al., 2019)
<i>Chlorella</i> sp and nappier grass	Anaerobic sludge	Batch	7 d, 30 d	39	409	448	8.7	(Jehlee et al., 2019)
<i>Chlorella</i> sp and empty fruit bunch	Anaerobic sludge	Batch	7 d, 30 d	22	466	488	4.5	(Jehlee et al., 2019)
Rubber sheet wastewater	Anaerobic sludge	Batch	7 d, 14 d	101	629	730	13.8	(Promnuan et al., 2020)
Mixed fruit waste	Anaerobic sludge	Batch	13 d, 50 d	61	208	269	22.6	(Basak et al., 2020)
Algal bloom biomass	Anerobic sludge	Batch	36 h, 7 d	39	274	313	12.5	(C. Chen et al., 2020)
Food waste	Anerobic sludge	Batch		37	307	344	10	(Ghimire et al., 2022)

<i>Food waste</i>	Anaerobic sludge	Batch	72h	0.6	2.36	2.96	20	(Sarkar et al., 2021)
<i>Food waste</i>	Anaerobic sludge	Batch	72 h	136	27	165	84	(Thi Nguyen et al., 2021)
<i>Starch wastewater</i>	Anaerobic sludge	Batch	72 h	63	184	247	25	(Warrajareansri & Wongthanate, 2021)
Continous mode of operation								
<i>POME</i>	POME biogas plant	CSTR, UASB	48 h, 15 d	3.8	14.4	20.2	20	(O–Thong et al., 2016)
<i>Food waste</i>	Anaerobic sludge	CSTR, FBR	3.7 d, 1.5 d	115	334	449	25.6	(An et al., 2024; Yeshanew et al., 2016)
<i>POME</i>	Anaerobic sludge	CSTR	3.75 d, 6.25 d	85	339	424	20	(Weng et al., 2018)
<i>sugarcane bagasse and water hyacinth</i>	Anaerobic sludge	CSTR	8 h, 10 d	27	92	119	22.7	(Kumari & Das, 2019)
<i>Food waste and paper waste</i>	Anaerobic sludge	CSTR	6 d, 24 d	78	373	451	17.3	(Qin, Li, et al., 2019)
<i>POME</i>	Anaerobic sludge	ASBR, UASB	2 d, 15 d	139#	454#	593	23.4	(Mamimin et al., 2017; Sivanesan et al., 2024)
Food waste	Anaerobic sludge	CSTR	2 d Single–stage	2.25	10.9	13.15	17.1	(Vo et al., 2019)
<i>Food waste</i>	Anaerobic sludge	CSTR	6 d, 24 d	50	426	476	10	(Qin, Wu, et al., 2019)
<i>Cellulosic wastewater</i>	Anaerobic sludge	CSTR	10 d	5.6	18.2	23.8	23.5	(Gadow & Li, 2020)
<i>Glucose</i>	Anaerobic sludge	Single–stage hybrid	12 h	53	271	324	16	(Yang et al., 2020)
<i>Sucrose</i>	Anaerobic sludge	CSTR	12 h	98	132	230	42	(D.–T. Ta et al., 2020)
<i>Pineapple waste</i>	Anaerobic sludge	CSTR	4 h, 72 h	15	80	95	15.6	(Chu et al., 2020)
<i>Sucrose</i>	Anaerobic sludge	CSTR	48 h	9	52	61	14.5	(D. T. Ta et al., 2020)
Rice straw and pig manure	Anaerobic digester sludge	CSTR	3 d, 27 d	16	197	213	7.5	(H. Chen et al., 2021)
Pilot scale								
Food waste	Anaerobic sludge	CSTR	3.3 d, 12.6 d	51	416	467	10.9	(Aashabharathi et al., 2024; Cavinato et al., 2011)

Municipal solid waste	Full scale AD reactor	CSTR– Biofilm reactor	7 d, 1 d	41	301	342	12	(Yeshanew et al., 2018)
<i>Municipal waste and sewage sludge</i>	Anaerobic sludge	CSTR	3 d, 17 d	24	570	594	4%	(Bolzonella et al., 2020)
POME		ASBR, UASB	48 h, 240 h	73	342	415	17.5	(Seengenyoun et al., 2019)
Corn stalk	–	CSTR, UASB	48 h, 24 h	25	95	120	20%	(Li et al., 2020)

Table 3. Microbiome involved in bio-hythane production.

Micro-organism	Major Fermentation products	Microbiome role and function	References
Major hydrogen producing bacteria			
<i>Clostridium</i> sp	H ₂ , acetate, butyrate,	Obligate anaerobic hydrogen producing bacteria utilizing wide range of organic waste including food waste, MSW, wastewater, <i>etc</i>	(Shanmugam et al., 2021b; Sivagurunathan et al., 2017)
<i>Enterobacter</i> sp	H ₂ , formate, acetate, lactate	Facultative anaerobic hydrogen producing bacteria, often detected in hydrogen producing granular reactor and having biofilm forming ability	(Hospital-Benito et al., 2024; Kotay & Das, 2010; Kumar et al., 2015)
<i>E.coli</i>	H ₂ , formate, acetate, lactate	Predominantly utilizes hexose and produces hydrogen via formate pathway	(Poladyan et al., 2020)
<i>Ethanoligenes</i> sp.	H ₂ , ethanol	Produces hydrogen via ethanol-mediated pathway	(Ren et al., 2008)
<i>Citrobacter</i> sp	H ₂ , ethanol, acetate	Able to ferment hexose with mixed acid fermentation pathway	(Das, 2017; Kotay & Das, 2010)
<i>Klbesiella</i> sp	H ₂ , lactate, ethanol, acetate, butyrate, formate	Able to ferment hexose with mixed acid fermentation pathway	(Mohammed et al., 2018)
<i>Sporolactobacillus</i> sp.	H ₂ , lactate, acetate, butyrate	Majorly lactic acid producing bacteria, often detected in hydrogen producing granular systems. Involved in the syntrophic relation with <i>Clostridium</i> sp for the biotransformation of lactate and acetate to butyrate and hydrogen formation.	(Park et al., 2020; Pugazhendhi et al., 2019)
Methane producing microbiome			
Hydrolytic and acidogenic consortia			
<i>Acetivibrio</i> , <i>Clostridium</i> , <i>Bacteroides</i> , <i>Ruminococcus</i> and <i>Thermotoga</i>		Involved in the hydrolysis and generation of shorty chain fatty acids	(Lim et al., 2020)
Non-hydrolytic acidogenes			

Bifidobacterium, Lactobacillus		Involved in the generation of shorty chain fatty acids without any hydrolytic activity	(Moestedt et al., 2020; Pokusaeva et al., 2011)
Syntrophic acetogenes			
<i>Smithllea</i> , <i>Syntrophobacter</i> and <i>Pelotomaculum</i>		Involved in the propionate oxidation and further transformation into acetate	(Venkiteshwaran et al., 2015)
<i>Syntrophus</i>		Involved in the oxidation of butyrate	(He et al., 2018)
<i>Syntrophomonas</i>		Involved in the oxidation of LCFA	(Kim et al., 2021)
<i>Methanogenes</i> , <i>Methanoculleus</i> , <i>Methanobacterium</i> , <i>Methanobrevibacter</i> , <i>Methanospirillum</i> and <i>Methanothermobacter</i>		Hydrogenotrophic methanogens	(Venkiteshwaran et al., 2015)
<i>Methanosaeta</i>	Its slow growth rate and great affinity for acetate allow it to spread and take over at low acetate concentrations. Its only substrate is acetate.	Obligate acetoclastic methanogens,	(Venkiteshwaran et al., 2015)
<i>Methanosarcina</i>	Capable of using a greater range of substrates, including methanol, aminomethane, methyl sulfides, and H ₂ as well as CO ₂ , in addition to acetate.	Facultative acetoclastic methanogens	(Venkiteshwaran et al., 2015)

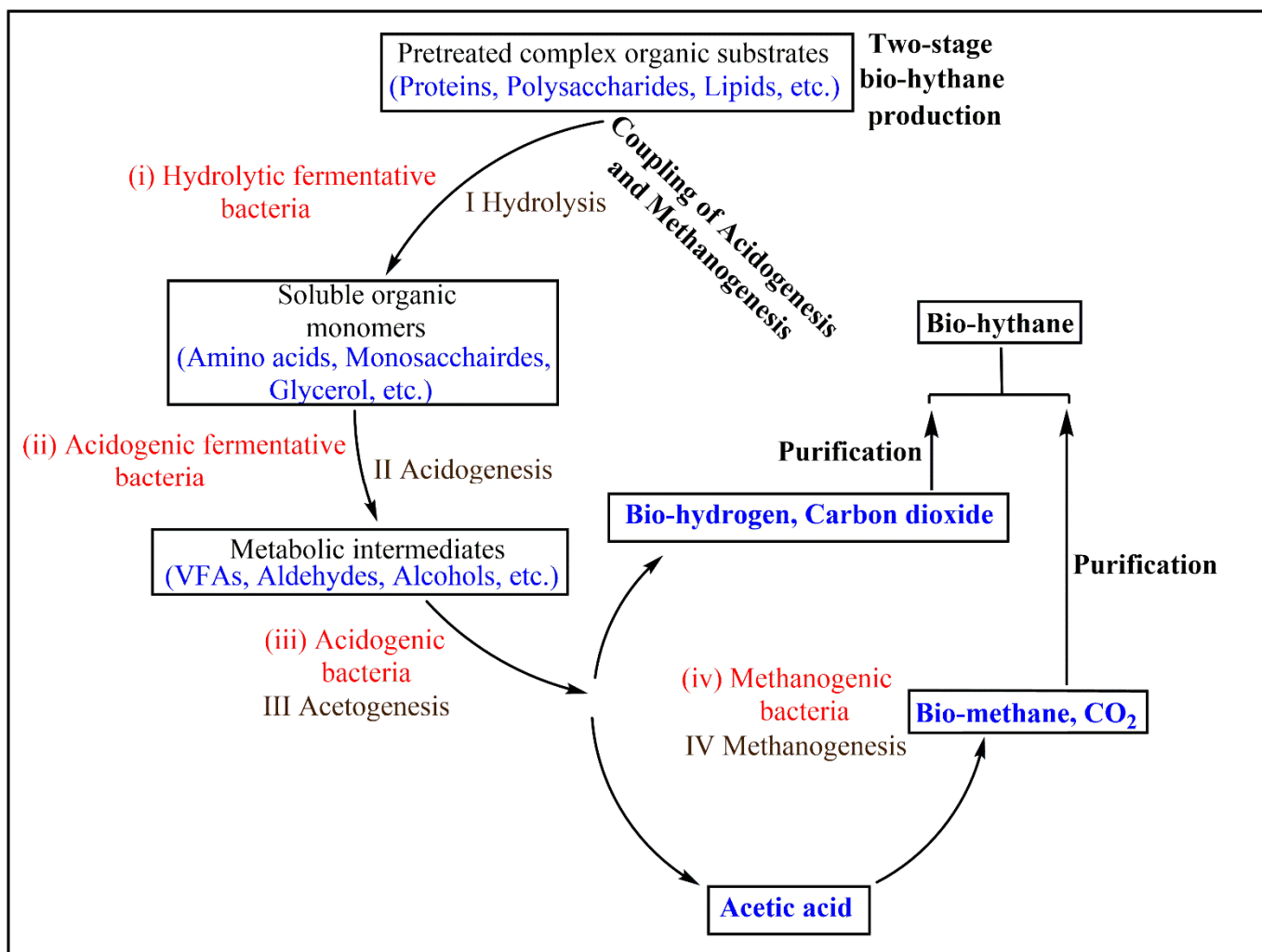


Fig. 1 Schematic representation of two step biohythane production.

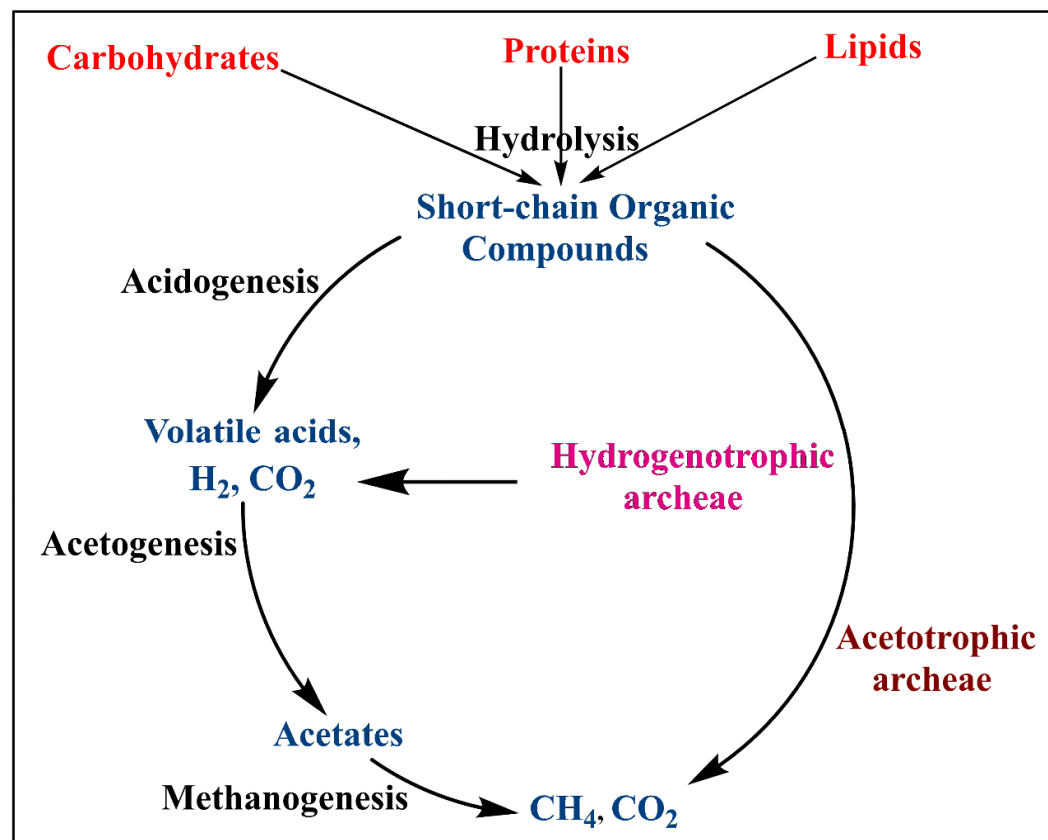


Fig. 2 Combination of acidogenesis and methanogenesis for production of bio-H₂ and bio-CH₄ (biohythane).

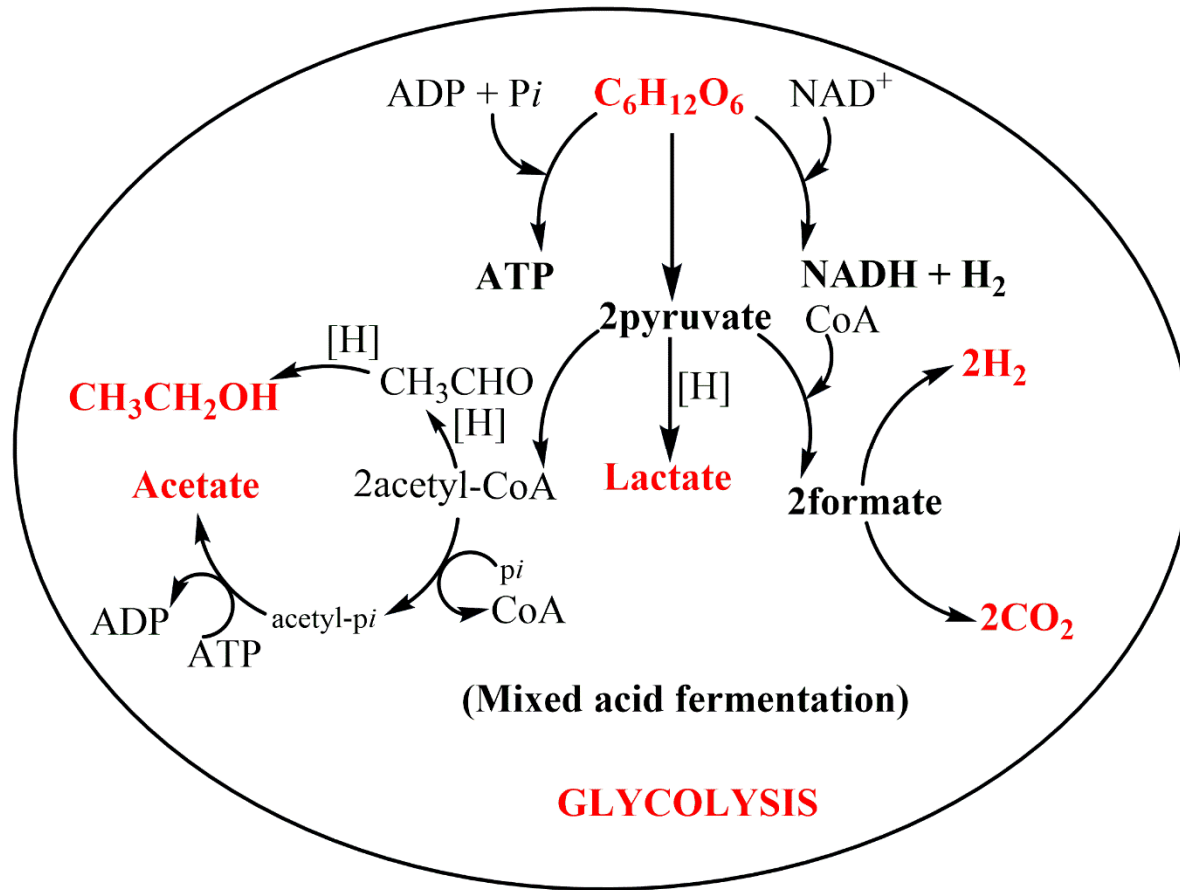


Fig. 3. Bio- H_2 production system with by-products by glycolysis.



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