Chapter 5

# Biohythane Production from Organic Wastes by Two-Stage Anaerobic Fermentation Technology

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Additional information is available at the end of the chapter

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**Abstract**

The combination of biohydrogen and biomethane production from organic wastes via two-stage anaerobic fermentation could yield a biohythane gas with a composition of 10-15% $H_2$, 50-55% $CH_4$ and 30-40% $CO_2$. Biohythane could be upgraded to biobased hythane by removing of $CO_2$. The two-stage anaerobic fermentation process is based on the different function between acidogens and methanogens in physiology, nutrition needs, growth kinetics, and sensitivity to environmental conditions. In the first stage, the substrate is fermented to $H_2$, $CO_2$, volatile fatty acids (VFA), lactic acid and alcohols by acidogens with optimal pH of 5–6 and hydraulic retention time (HRT) of 1–3 days. In the second stage, the remaining VFA, lactic acid, and alcohols in the $H_2$ effluent are converted to $CH_4$ and $CO_2$ by methanogens under optimal pH range of 7–8 and HRT of 10–15 days. The advantage of biohythane over traditional biogas are more environmentally, flexible of $H_2/CH_4$ ratio, higher energy recovery, higher degradation efficiency, shorter fermentation time, and high potential to use as vehicle fuel. This chapter outlines the general approach of biohythane production via two-stage anaerobic fermentation, principles, microorganisms, reactor configuration, process parameters, methods for improving productivity as well as technical challenges toward the scale-up process of biohythane process.

**Keywords:** biohythane, microbiology and biochemistry, physicochemical parameters, reactor configuration, improvement methods, two-stage anaerobic fermentation, organic wastes

## 1. Introduction

Currently, development of biofuels to replace fossil fuels by the biological process has been attracting attention as an environmentally friendly process. Among the various processes, biohydrogen and biohythane are the promising future energy carriers due to their potentially higher conversion efficiency and low pollutants generation [1]. Dark fermentation shows high $H_2$ production rate under realistic conditions, which is approaching practical levels [2]. In addition, the major advantages are rapid bacterial growth rates, relatively high $H_2$ production capacities, operation without light sources, no oxygen limitation problems, and low capital cost of at least at small-scale production facilities [3, 4]. The dark fermentation process can utilize organic materials for $H_2$ gas production, such as cellulose and starch-containing agricultural and food industry wastes, and some food industry wastewaters, such as cheese whey, olive mill, palm oil mill, and baker's yeast industry wastewaters [5]. $H_2$ yields from dark fermentation of organic wastes such as food waste, apple processing wastewater, starch wastewater, palm oil mill effluent, and potato processing wastewater were 57, 92, 92, 115, and 128 mL $H_2$/gCOD, respectively [6–9]. However, dark fermentation has low substrate conversion efficiency as only 7.5–15% of the energy contained in organic wastes are converted to $H_2$ and the rest of the energy still remains in the liquid ($H_2$ effluent) as VFA (mainly butyric acid and acetic acid), lactic acid, and alcohols [1]. The disadvantage of dark fermentation must be overcome before biohydrogen can become economically feasible. The conversion of VFA, lactic acid, and alcohols to $CH_4$ through anaerobic digestion (AD) [10] is faster and simpler than the conversion of these components to $H_2$ by photo-fermentation and microbial-electrolysis process [1]. In addition, it has been shown to be an energy efficiency strategy for the production of a mixture of $H_2$ and $CH_4$, known as biohythane, via two-stage anaerobic fermentation [11, 12].

Biohythane has attracted growing attention worldwide due to its potential use as vehicle fuel, high potential to produce from conversion of organic wastes and probably an alternative to the fossil-based hythane [10]. Normally, hythane gas was produced from a thermo-chemical process using natural gas as a starting material. This process is a high-energy consumption and still depends on fossil fuel. Biohydrogen and biomethane production from organic wastes by fermentation process and anaerobic digestion process, respectively, are already established. The combination of these two processes via two-stage anaerobic fermentation processes could yield a $H_2$ and $CH_4$ gas with a composition like hythane (10–15% $H_2$, 50–55% $CH_4$, and 30–40% $CO_2$) called biohythane [13], which could be upgraded to biobased hythane by removing of $CO_2$. The two-stage anaerobic fermentation for biohythane production is involved with the fermentation of organic wastes to $H_2$, $CO_2$, VFA, lactic acid, and alcohols in the first stage and conversion of these substances in $H_2$ effluent to $CH_4$ and $CO_2$ via anaerobic digestion process in the second stage (**Table 1**). The optimum condition for the first stage is a pH range between 5 and 6 and a hydraulic retention time (HRT) range of 1–3 days that are suitable for acidogens for the conversion of organic wastes to $H_2$ via the acetate and butyrate pathways. In the second stage, the acetic acid in the $H_2$ effluent is converted to $CH_4$ and $CO_2$ by acetoclastic methanogens under an anaerobic condition with optimal pH range of 7–8 and optimal HRT of 10–15 days [11]. Others VFA, lactic acid, and alcohols in the $H_2$ effluent are anaerobically converted by acetogens to $H_2$ and $CO_2$, which are consequently converted to $CH_4$ by hydrogenotrophic methanogens [14].

| Technology | Processes | Substrates | Products |
|---|---|---|---|
| Hythane | Thermo-chemical | Natural gas | 5–7% $H_2$, 90% $CH_4$ and 5% $CO_2$ |
| Biomethane | Anaerobic digestion (AD) | Organic wastes | 50–60% $CH_4$ and 40–50% $CO_2$ |
| Biohydrogen | Fermentation | Organic wastes | 40–60% $H_2$ and 40–60% $CO_2$ |
| Biohythane | Two-stage fermentation/AD | Organic wastes | 5–10% $H_2$, 60% $CH_4$ and 30% $CO_2$ |

**Table 1.** Biohythane technology development from two-stage anaerobic fermentation technology.

The two-stage anaerobic fermentation process could increase energy recovery, degradation efficiency, reactor stability, $CH_4$ production rates, and purity of gas products when compared to one-stage $H_2$ or $CH_4$ fermentation [15]. In addition, the two-stage process has advantages of improving negative impacts of inhibitive compounds in feedstocks (such as wheat hydrolysate, molasses, and skim latex serum), operated at high organic loading rates and reduced fermentation time with total HRT of 10–18 days for overall processes. Advantages of biohythane over traditional biogas are improved energy recovery, shortened fermentation time, flexible $H_2/CH_4$ ratio, and more environmentally benign and process robustness for handling the organic wastes [10, 16]. Integrated biohydrogen with biomethane process worth for commercialization could get the biogas in the form of biohythane. Typically, the suggested $H_2$ content in biohythane is 10–15% by volume. Biohythane is considered to be a clean fuel for vehicles compared to gasoline or diesel due to low greenhouse gas emission from the combustion process [17].

Biohythane via two-stage anaerobic fermentation using organic wastes could be a promising technology for higher energy recovery and cleaner transport biofuel than biogas. Various types of organic wastes can be used as substrate for biohythane production such as starch wastewater, wheat straw hydrolysate, palm oil mill effluent, food waste, and organic solid waste [13, 18–20]. Wheat straw hydrolysate was used for biohythane production by *Caldicellulosiruptor saccharolyticus* with maximum $H_2$ production rate of 5.2 L $H_2$/L·d and maximum $CH_4$ production rate of 2.6 L $CH_4$/L·d. The maximum energy output of the process was 10.9 kJ/g of straw with energy recovery of 57% of energy contained in the wheat straw [20]. Biohythane production of starch wastewater achieved $H_2$ and $CH_4$ yields of 130 mL $H_2$/gCOD and 230 mL $CH_4$/gCOD, respectively [18]. Biohythane production of food waste achieved $H_2$ and $CH_4$ yields of 205 mL $H_2$/gVS and 464 mL $CH_4$/gVS, respectively [21]. Biohythane production of palm oil mill effluent (POME) was achieved with $H_2$ and $CH_4$ yields of 201 mL $H_2$/gCOD and 315 mL $CH_4$/gCOD, respectively [13]. Nathao et al. [22] obtained two-stage process for biohythane production from food waste with $H_2$ and $CH_4$ yields of 55 and 94 mL/gVS at F/M of 7.5. Kongjan et al. [11] used UASB reactors for extreme thermophilic $H_2$ and thermophilic $CH_4$ production from wheat straw hydrolysate via a two-stage anaerobic fermentation process. Specific $H_2$ and $CH_4$ yields of 89 mL $H_2$/gVS and 307 mL $CH_4$/gVS, respectively, were achieved. Successful continuous biohythane production from POME by two-stage thermophilic fermentation and mesophilic anaerobic digestion was reported by Mamimin et al. [13]. The continuous biohythane production rate of 4.4 L/L·d was achieved with biogas containing 51% $CH_4$, 14% $H_2$, and 35% $CO_2$. Energy analysis suggested that the two-stage fermentation process for biohythane production had greater net energy recovery than the single $H_2$ fermentation and $CH_4$ fermentation process. This chapter provides the information on general approach of biohythane via two-stage anaerobic fermentation, principles of biohythane process, microorganisms involved in $H_2$ and $CH_4$ production, reactor configuration for biohythane production, methods for improve biohythane production, process parameters affecting biohythane production and technical challenges toward the scale-up process.

## 2. Principles of biohythane process

Most of wastewater and organic wastes were usually treated in an anaerobic process for $CH_4$ recovery as energy. Regarding clean energy of $H_2$, anaerobic process was modified for $H_2$ production by suppression of methanogenic activity. To harvest $H_2$ from the first stage, the $H_2$-consuming pathway has to be inhibited [23]. Most $H_2$-producing bacteria can form endospores in stress environment. Various selection methods can be used to enrich $H_2$-producing bacteria [24]. The most common selection methods are heat treatment and pH control. However, some researchers reported the invalidity of such selection methods [25], because not all $H_2$-producing bacteria are associated with the ability to form endospores. In addition, there are many $H_2$-consuming bacteria that can form endospores, such as acetogens and sulfate-reducing bacteria [26]. The pH control is an important method for maintaining $H_2$-producing bacteria in continuous systems of first stage. The pH varies depending on the microbial species, microbial activities, reactor configuration, feedstock characteristics, organic loading rate, buffer capacity, and temperature. The change of pH is due to acetic acid and butyric acid production accompanies with $H_2$ production, whereas the low pH influences on the shift of metabolic products from acidogenesis to solventogenesis [27]. Low pH is also critical strategies to inhibit the activity of methanogenesis. The suggestion for optimal pH of $H_2$ production could range from 5.0 to 6.5. From the perspective of thermodynamics, changes of Gibbs free energy during $H_2$ production were much larger than those of methanogenesis. This means faster rates for microbial growth in biohydrogen fermentation. On the basis of this characteristic, the manipulation of hydraulic retention time (HRT), temperature, and oxidation-reduction potential (ORP) can achieve microbial $H_2$ process feasible in continuous operation.

Continuous biohythane production by integrating biohydrogen with biomethane process worth for commercialization could get the biogas that has composition like hythane gas. In the first stage, substrate is fermented to $H_2$, $CO_2$, VFA, lactic acid, and alcohols whereby the non-gas metabolites are converted to $CH_4$ and $CO_2$ in the second stage [10]. The fermentation products from $H_2$ production process are very important for the whole biohythane system performance because they can affect the loading, degradation efficiency, and operating stability of the methanogenesis stage [28]. The conversion rate from VFA to acetic acid will affect the methanogenic archaea quantity, and subsequently affect the degradation rate of acetic acid and $CH_4$ yield. The basic principle of a two-stage process is shown in **Figure 1**. The first stage includes hydrolysis and acidogenesis where hydrolytic and fermentative bacteria excrete enzymes to break down complex organic compounds of carbohydrate, protein, and lipid into single molecules of mono sugar, amino acid, and long chain fatty acids and/or glycerol respectively. The acidogenesis, fermentative, and acidogenic bacteria convert the hydrolysis products into $CO_2$,

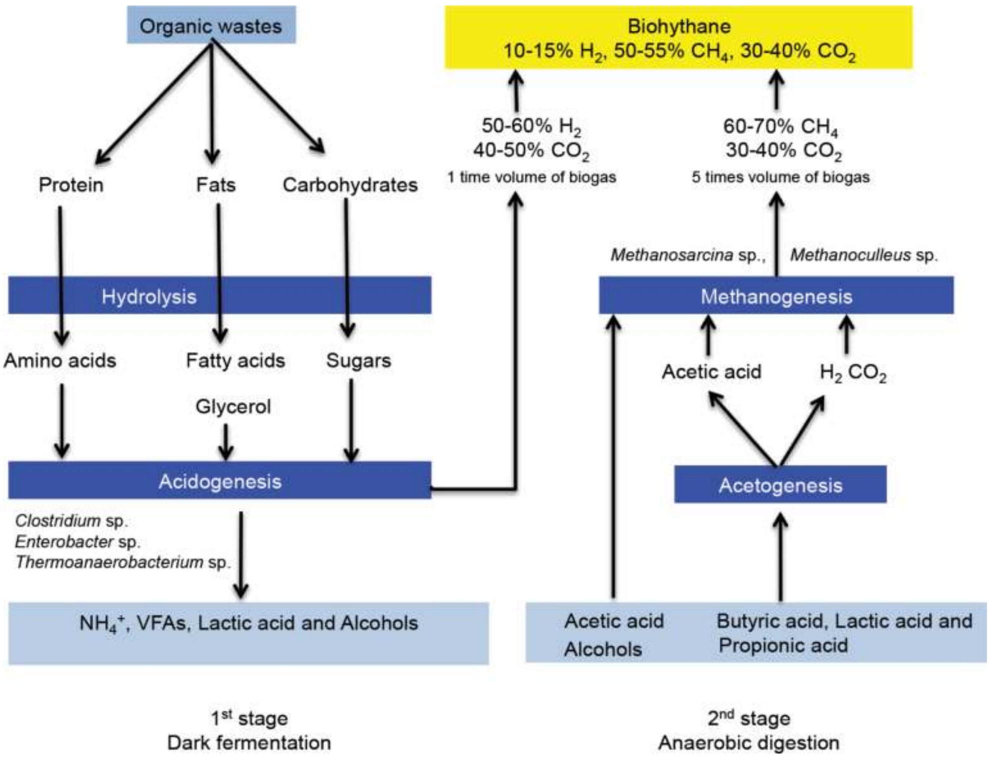


**Figure 1.** Modification of anaerobic digestion for biohythane production from organic wastes via two-stage anaerobic fermentation process.

$H_2$, VFA, lactic acid, and alcohols. High $H_2$ production was achieved by fermentative bacteria via acidogenesis process under pH range of 5-6 and operating at short HRT of 1-3 days. Under the optimum condition, acidogenic bacteria could convert carbohydrate to $H_2$ and $CO_2$ via the acetate and butyrate pathways and competition to other microorganisms. In the second stage, the acetic acid in the $H_2$ effluent is anaerobically converted to $CH_4$ and $CO_2$ by acetoclastic methanogens. The acetogenic bacteria could produce acetic acid along with additional $H_2$ and $CO_2$ from butyric acid, propionic acid, and lactic acid. $H_2$ and $CO_2$ are consequently converted to $CH_4$ by hydrogenotrophic methanogens [29]. These reactions occur under an optimal pH range of 7–8 and HRT of 10–15 days [30]. The two-stage anaerobic fermentation process is also characterized by a significantly reduced fermentation time with overall fermentation time of 13–18 days [10].

The two-stage anaerobic fermentation process is based on two physiologically different groups of microorganisms. One group of acidogenic bacteria that converts organic matter into $H_2$, $CO_2$, soluble VFA, lactic acid, and alcohols, is fast growing, prefers a slightly acidic environment of pH 5–6, and is less sensitive to environmental changes. A large number of microbial species, including strict and facultative anaerobic bacteria such as *Clostridium* sp., *Enterobacter* sp., *Caldicellulosiruptor* sp., *Thermotoga* sp., and *Thermoanaerobacterium* sp., are efficient $H_2$ producers, while degrading various types of carbohydrates [31]. The other group in second stage is methanogenic archaea, which converts VFA, lactic acid, and alcohols into $CH_4$ and $CO_2$, is slow growing, prefers neutral to slightly alkaline environments, and is very sensitive to environmental changes. *Methanosarcina* sp. and *Methanoculleus* sp. were dominant and played an important role in second stage [14, 15]. *Methanosarcina* species were reported to be dominant at high acetate concentration (>1.2 mM), and the results were consistent with the high acetate concentrations in $H_2$ effluent that feed to $CH_4$ reactors. *Methanoculleus* species were responsible for hydrogenotrophic methanogenesis that convert $H_2$ and $CO_2$ to $CH_4$ [11]. Obtaining the optimum environmental conditions for each group of organisms by the two-stage anaerobic fermentation process provides several advantages over the conventional single stage [32–34], e.g., high net energy efficiencies, more stable operation, allowing higher organic loading rate operation, smaller-size reactor (40–60% smaller), thus better economics for construction cost and higher $CH_4$ content in the biogas (65–75%) [15, 35]. High $CH_4$ content and production was found in the second stage due to $CO_2$ in the second stage is mainly generated by aceticlastic methanogen and then consumed partly by hydrogenotrophic methanogen also existed in the second stage. The higher $CH_4$ content is definitely a better fuel value for on-site use and higher digestion efficiency, thus more $CH_4$ is recovered [36].

## 3. Microorganisms in biohythane process

The two-stage anaerobic fermentation process is based on the differences between acidogens and methanogens in physiology, nutrition needs, growth kinetics, and sensitivity to environmental conditions. The acidogens and methanogens are enriched separately in two tanks enabling optimized growth by maintaining proper environmental conditions in each reactor [37]. Microorganisms involved in the first stage $H_2$ production and in the second stage $CH_4$ production via two-stage anaerobic fermentation process are shown in **Table 2**. First stage ($H_2$ reactor) involved with the several bacterial strains is capable to produce $H_2$ through dark fermentation of various carbohydrates. Obligate anaerobic *Clostridia* are potential $H_2$ producers and are well known for high $H_2$ yield [38]. *C. butyricum*, *C. welchii*, *C. pasteurianum*, and *C. beijerinckii* were used for $H_2$ production [39]. *Clostridium* sp. is capable of utilizing a wide range of carbohydrates such as xylose, arabinose, galactose, glucose, cellobiose, sucrose and fructose with a $H_2$ yield of 2.1–2.2 mol $H_2$/mol sugars [40]. Facultative anaerobes *Enterobacteriaceae* are $H_2$ producers that are resistant to trace amount of dissolved oxygen. *Enterobacter* sp. has lower yield (1.0 mol $H_2$/mol sugars) when compared to *Clostridium* sp. [41]. *Citrobacter* sp. also belongs to family *Enterobacteriaceae* known to produce $H_2$ from CO and $H_2O$ by water-gas shift reaction under anaerobic condition [42]. *Escherichia coli* is capable of producing $H_2$ and $CO_2$ from formate in the absence of oxygen. The $H_2$ yields of *E. coli* were 0.6–1.3 mol $H_2$/mol glucose [43]. *Bacillus* sp. also has been identified as $H_2$ producers such as *B. licheniformis* [44] and *B. coagulans* [45]. Its $H_2$ yield was 0.5 mol $H_2$/mol glucose with lactic acid as main soluble metabolites. Dark fermentation at thermophilic temperatures (55–60°C) showed favorable kinetics and stoichiometry of $H_2$ production compared to the mesophilic systems. Metabolism at higher temperatures becomes thermodynamically more favorable

| Stages | Mesophilic condition (30–35°C) | Thermophilic condition (55–60°C) | Extreme thermophilic condition (70–90°C) |
|---|---|---|---|
| 1st hydrogen production (Bacteria) | *Clostridium* sp.<br>*Enterobacter* sp.<br>*Citrobacter* sp.<br>*Bacillus* sp. | *Thermoanaerobacterium* sp.<br>*Clostridium* sp.<br>*Thermoanaerobacter* sp. | *Caldanaerobacter* sp.<br>*Caloramator* sp.<br>*Thermotoga* sp. |
| 2nd methane production (Bacteria) | *Clostridium* sp.<br>*Bacillus* sp.<br>*Desulfobacterium* sp. | *Clostridium* sp.<br>*Thermoanaerobacterium* sp.<br>*Desulfomicrobium* sp. | *Caloramator* sp. |
| 2nd methane production (Archaea) | *Methanobacterium* sp.<br>*Methanoculleus* sp.<br>*Methanospirillum* sp.<br>*Methanococcus* sp.<br>*Methanobacter* sp. | *Methanothermobacter* sp.<br>*Methanosarcina* sp. | *Methanothermus* sp.<br>*Methanothermococcus* sp. |

**Table 2.** Microorganisms involved in the first stage $H_2$ production, and the second stage $CH_4$ production via two-stage anaerobic fermentation process.

and less affected by the partial pressure of $H_2$ in the liquid phase. Dark fermentation under thermophilic condition was involved with *Thermoanaerobacterium* sp., *Thermoanaerobacter* sp., and *Clostridium* sp. [15]. *Thermoanaerobacterium thermosaccharolyticum* has an optimal growth at moderate thermophilic temperature (60°C) and can convert carbohydrate to $H_2$ via butyrate- and acetate-type fermentation [46]. *Thermoanaerobacterium* species are well known as good $H_2$-producing bacteria [8, 47]. *Thermoanaerobacterium* sp. represents anaerobic spore forming thermophilic microorganisms previously found in thermophilic $H_2$-producing reactors [8, 9]. Genus *Thermoanaerobacterium*, especially *Tbm. thermosaccharolyticum*, is capable of $H_2$ production from various types of substrate under the thermophilic conditions. Various *Tbm. thermosaccharolyticum* strains have been isolated such as strain PSU2 [46], strain GD17 [48], strain W16 [49], strain KKU19 [50], and strain IIT BT-ST1 [51]. In addition, *Tbm. thermosaccharolyticum* can grow on various organic wastes including hemicellulosic waste and lignocellulosic waste [48, 52]. *Thermoanaerobacter* sp. has optimal growth at moderate thermophilic temperature (60°C) and can convert carbohydrate to $H_2$ via ethanol- and acetate-type fermentation, but cannot degrade cellulose. These species produce $H_2$, ethanol, lactate, acetate, and $CO_2$ as the major products, but no butyrate production. Thermophilic *Clostridium* sp. was found to degrade cellulose using cellulase enzymes and can ferment the lignocellulosic biomass to $H_2$ with the yield of 1.6 mol $H_2$/mol hexose [53]. Dark fermentation at extreme thermophilic temperatures (70–90°C) showed more favorable kinetics and stoichiometry of $H_2$ production compared to the thermophilic and mesophilic systems. Dark fermentation under extreme thermophilic condition was involved with *Thermotoga* sp. and *Caldicellulosiruptor* sp. [54]. The $H_2$ production ability of *Caldicellulosiruptor* sp. was explored at extreme temperatures. These microbes are known to have various kinds of hydrolytic enzymes that can utilize a wide range of substrate such as cellulose, cellubiose, and xylan. *Caldicellulosiruptor* sp. has high potential to use lignocellulosic waste for $H_2$ production with the yield of 3.3 mol $H_2$/mol hexose. The predominant metabolites formed by these organisms are acetic acid and lactic acid [55]. *Thermotoga* sp. was isolated from geothermal spring and capable to grow and produce $H_2$ at temperatures of 90°C. *Thermotoga* sp. can use elemental sulfur as electron source with $H_2$ yield of 3.5 mol $H_2$/mol hexose [56]. The soluble metabolites of these strains are mostly acetic acid, $H_2$, $CO_2$, and trace amount of ethanol [57].

Microbial consortium or mixed cultures are providing more enzymes for the utilization of complex substrate than pure cultures. Mixed microbial consortium can be developed from various sources such as anaerobic digested sludge, soil samples, and wastewater by heat treatment and load-shock treatment [58]. These two treatments could eliminate unwanted microorganisms such as methanogens and $H_2$-consuming bacteria while enriching an $H_2$-producing bacterium. Heat treatment inhibits the activity of the methanogens and $H_2$ consumers, while the spore forming $H_2$-producing bacteria was survived. Additionally, continuous operation at a low hydraulic retention time (1–2 days) helps in washing out slow-growing methanogens from $H_2$ reactor. Industrially, the use of mixed cultures for $H_2$ production from organic wastes in the first stage could be more advantage than pure cultures. Enriched $H_2$-producing bacteria from anaerobic sludge could utilize cellulose as a substrate for $H_2$ production with the yield of 2.4 mol $H_2$/mol hexose [59]. The fermentation of various organic wastes by mixed cultures gave the $H_2$ yields in the range of 57–128 mL $H_2$/gCOD, depending on type of waste [6–9]. This indicates the practical potential to commercialize $H_2$ production from organic wastes by mixed microbial consortium.

The second stage $CH_4$ reactor involved with several archaea strains is capable to produce $CH_4$ through anaerobic fermentation of VFA, lactic acid, and alcohols. The order *Methanobacteriales* comprises of two families (*Methanobacteriaceae* and *Methanothermaceae*) is $CO_2$, $H_2$, and methanol consuming methanogens. The family *Methanobacteriaceae* including *Methanobacterium* sp., *Methanothermobacter* sp., *Methanobrevibacter* sp., *Methanothermus* sp., and *Methanospaera* sp. are commonly found in $CH_4$-producing reactor. *Methanothermobacter* sp. is a thermophilic *Methanobacteriaceae* that is commonly found in thermophilic $CH_4$-producing reactor. *Methanothermus* sp. is an extreme thermophilic *Methanobacteriaceae* that is commonly found in extreme thermophilic $CH_4$-producing reactor. *Methanothermus* sp. grows at a temperature of 83–85°C and assimilates $CO_2$ and $H_2$ [60]. The order *Methanococcales* consists of *Methanocaldococcus* sp., *Methanothermococcus* sp., and *Methanococcus* sp. These archaea produces $CH_4$ from $CO_2$ and $H_2$ or formate as the energy source. [61]. The order *Methanomicrobiales* consists of *Methanomicrobium* sp., *Methanocorpusculum* sp., *Methnanoplanus* sp., *Methanospirillum* sp., and *Methanoculleus* sp. These archaea produce $CH_4$ from acetic acid and exception of *Methanocorpusculum* sp. and *Methanoculleus* sp. using $CO_2$ and $H_2$ for $CH_4$ production [62]. The order *Methanosarcinales* consists of *Methanosarcina* sp., *Methanohalobium* sp., *Methanohalophilus* sp., *Methanolobus* sp., and *Methanosaeta* sp. *Methanosarcina* sp. are hydrogenotrophic or acetoclastic and thus can reduce $CO_2$ to $CH_4$ or can utilize acetic acid to $CH_4$ and $CO_2$. *Methanosarcina* sp. also can convert methyl-group-containing compounds such as methanol, methylamines, and methyl sulfides to $CH_4$ and $CO_2$. *Methanosaeta* sp. utilizes acetic acid as the energy source through acetoclastic reaction.

Acidogenic $H_2$ producers grow faster than methanogens and eventually produce VFA in effluent. Major genuses related to acidogenic $H_2$ production are *Enterobacter* sp., *Clostridium* sp., *Citrobacter* sp., *Thermoanaerobacterium* sp., and *Caldicellulosiruptor* sp. After $H_2$ production, effluents rich in VFA such as acetic acid, butyric acid, lactic acid, and ethanol would be consumed by methanogenic archaea at neutral pH. High acetic acid concentration promotes the growth of *Methanosarcina* sp. On the contrary, lower acetic acid concentration is preferred by *Methanosaeta* sp. For acetoclastic methanogens such as *Methanosarcina* sp., the minimum thresholds for acetate utilization are typically in the range of 0.5 mM and higher. The minimum thresholds for acetic acid utilization of *Methanoseata* sp. are in the micromole range. The presence of *Clostridium*, *Bacillus*, and *Desulfobacterium* in $CH_4$ production stage is in accordance with the significant removal of lactic acid in the $H_2$ effluent since *Clostridium* and *Desulfobacterium* spp. are able to degrade lactic acid to acetate and/or $H_2$ [63]. Meanwhile, some acidogenic bacteria, *Thermoanaerobacterium* sp., *Clostridium roseum*, and *Clostridium isatidis*, which are $H_2$ producers [64–66] were also detected in $CH_4$ stage, confirming that some $H_2$ and $CO_2$ were also produced. However, the presence of the hydrogenotrophic methanogens of *Methanothermobacter defluvii* and *Methanothermobacter thermautotrophicus* could possibly consume $H_2$; thus, no $H_2$ could be detected when the methanogenic stage reached stable conditions [67].

## 4. Process parameters affecting biohythane production

Biohythane production processes are greatly influenced by complex biochemical and physical parameters. The process parameters such as inoculum properties, complexity of substrate, nutrient, alkalinity, $H_2$ concentration, hydraulic retention time (HRT), and toxic compounds have influence on biohythane process (**Table 3**). Inoculums and feedstocks compositions greatly affect first stage $H_2$ fermentation when using mixed cultures and non-sterile feedstocks [1, 70, 74]. Environmental and physical factors greatly affect the second stage $CH_4$ production [75, 76]. To stabilize and maximize $H_2$ production, it is necessary to direct the metabolic pathway toward acetic acid and/or butyric acid and also to maintain the right $H_2$-producing bacteria during first stage operation. The performance of microorganisms in the conversion of substrate to $H_2$ is also dependent on the efficiency of its enzymatic machinery. The main factors affecting two-stage anaerobic fermentation are described as follows.

### 4.1. Feedstocks

Biohythane can be produced from various substrates mainly carbohydrate. In terms of $H_2$ rate and yields, carbohydrates are the most suitable feedstock followed by protein and peptides, while fat is considered very limited [77]. Most of dark fermentation for $H_2$ production has been conducted with glucose or sucrose. Glucose is the monomeric unit of cellulose and starch which is a major component in organic wastes [78]. Carbohydrate-rich organic waste is a favorable substrate for $H_2$ fermentation [79, 80]. The $H_2$ yield from bean curd manufacturing waste was significantly low compared to carbohydrate-rich substrates [80]. For stable $H_2$ fermentation, a carbon/nitrogen (C/N) ratio of feedstock greater than 20 is recommended [81]. The $H_2$ fermentative microorganisms showed improvement in $H_2$ production when they were grown in a fermentation media having a C/N ratio greater than 20. The C/N ratio of 20–30 also has positive effect on $CH_4$ production stage. Phosphate concentration in feedstock is also

| Factors | Effects on biohythane process | References |
|---|---|---|
| Feedstocks | • Fermentation metabolism, microbial activity, and microbial community | [68] |
| Inoculum | • Fermentation metabolism and microbial community | [69] |
| pH and Alkalinity | • Fermentation metabolism, microbial activity, and microbial community<br>• Cell membrane charge<br>• Metabolic shift to solvent production | [70] |
| Temperature | • Fermentation metabolism, microbial activity, and microbial community | [71] |
| HRT | • Fermentation metabolism, microbial activity, and microbial community<br>• Microbial growth rate | [72] |
| $H_2$ Partial Pressure | • Fermentation metabolism and activity<br>• Activity of acetogens and methanogens | [70] |
| Trace element | • Essential for cell growth,<br>• Enzyme activity | [73] |

**Table 3.** Main factors affecting the two-stage anaerobic fermentation for biohythane production from organic wastes.

important in dark fermentation. Phosphate helps in maintaining buffered condition during fermentation and provides the building blocks of nucleic acid and ATPs. In dark fermentation, an increase in phosphate concentration leads to enhancement of the $H_2$ production [47].

### 4.2. Inoculums

Developing an enriched inoculum is very important for obtaining $H_2$ in first stage fermentation. In the enrichment process, selection procedure was applied to selectively promote $H_2$-producing bacteria and eliminate $H_2$ consumers. Different selective procedures such as heat, acid, ultrasonic, ultraviolet, organic and alkali treatment were commonly used [58]. Most of $H_2$-producing bacteria are spore forming, while $H_2$-consuming bacteria and methanogens are non-spore forming, which get eliminated with selection methods. The selection methods are promoting endospores formation in a certain group of bacteria that also include $H_2$-producing bacteria. Thus, under favorable conditions, the endospores germinate and the $H_2$-producing bacteria dominate in the system. The $H_2$-producing inoculum might consist of sporulating bacteria like *Bacillus* sp. and *Clostridium* sp. Furthermore, the bacteria capable of producing $H_2$ widely exist in natural environment in the form of mixed cultures such as anaerobic sludge, municipal sewage sludge, hot spring sediment, compost and soil have been widely used as inoculum for fermentative $H_2$ production [82–84]. Using mixed cultures is more practical than using pure cultures due to the easy operating and control under the non-sterile condition. Mixed cultures also have a broader source of feedstock [85]. The selection of $H_2$-producing bacteria suitable for introduction into $H_2$ reactor may be regarded as inoculum preparation. It should consider the revival of bacteria from the stock, successive of subculturing to active bacteria, short lag phase and high active

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cells [86]. Inoculum size for dark $H_2$ fermentation was varied in the range of 10–20% (v/v). This depends on the characteristics of the species and medium used. Obligate anaerobes produce very less amount of biomass; thus, larger inoculum volume and concentration are required. The inoculum age also matters during the fermentation. Cells growing at the exponential phase have the entire enzymatic machinery active which is required for $H_2$ and $CH_4$ production.

### 4.3. Hydrogen partial pressure

The $H_2$ partial pressure in the liquid phase is the major factor affecting $H_2$ production, as high $H_2$ partial pressure causes deactivation of hydrogenase enzyme. Decreasing $H_2$ partial pressure by intermittent nitrogen sparging of batch reactor headspace could enhance $H_2$ production during thermophilic fermentation [87]. In addition to a high $H_2$ partial pressure, the NADH, which is an electron carrier in the cell, will be oxidized mainly to lactate during extreme thermophilic fermentation with *Caldicellulosiruptor saccharolyticus* [88]. The formation of lactate during the overloading or unstable conditions might be caused by a high $H_2$ partial pressure.

### 4.4. Hydraulic retention time (HRT)

The total time that cells and soluble nutrients reside in the reactor is called the HRT. $H_2$ production occurring at low HRT is dependent on the volume of the reactor and the flow rate of feed. It is generally well known that the $H_2$-producing bacteria are fast growing [70]. By applying this principle, Liu et al. [48] produced $H_2$ free of $CH_4$ in continuously CSTR feeding with household solid waste at acidic pH range of 5.0–5.5 and a short HRT of 3 days without any pretreatment to inhibit methanogens contained in the initial digested manure. HRT is the main optimization parameters of continuous $H_2$ dark fermentation bioprocesses. In the CSTRs, short HRTs or high dilution (D) rates can be used to eliminate methanogens, which have significant low growth rate [70, 89]. However, HRT is needed to be maintained in a proper level that still gives a D value less than specific growth rate of $H_2$-producing bacteria. Generally, short HRT is considered to favor the $H_2$ fermentation metabolism [3]. On the other hand, too high loading rates may result in substrate inhibition effects, improper food to microorganism (F/M) ratios of $H_2$ producers or washout of microorganisms [90]. These shock loads could reduce the $H_2$ production metabolism through decreasing of pH and metabolite inhibition (accumulation of intermediates). The HRT could also help in the enrichment of microbial consortium, since it directly affects the specific growth rate of bacteria. By manipulating the HRT, slow-growing microbes like methanogens and $H_2$-consuming microbes can be expelled out of the reactor, thus leading to selective enrichment of $H_2$-producing bacteria [91]. This approach of using short HRT for suppressing methanogens led to improvement in $H_2$ production [92]. In second stage, the HRT is a measure to describe the average time that a certain substrate resides in a digester. If the HRT is shorter, the system will fail due to washout of microorganisms. HRT for anaerobic digestion process are typically in the range of 15–30 days at mesophilic conditions and 10–20 days at thermophilic conditions [13]. Long retention times also benefit hydrolysis of the particulate matter of complex structure such as lignocellulose biomass [93]. On the other hand, organic loading rate (OLR) or amount of organic matter in the system is relative with HRT. The shorter HRT will achieve high OLR that leads to the accumulation of VFA which consequently leads to a pH drop and inhibition of methanogenic activity. This causes a system failure. During methanogenesis, the HRT should be kept two-fold greater than the generation time of the slow-growing microbes [94]. The HRT should be held for a suitable duration so that the dead zones get eliminated, and it would also help in promoting an efficient syntrophy among the microorganisms present in the mixed culture.

### 4.5. pH and alkalinity

Among all the chemical factors influencing dark fermentation, pH is considered the most influential. It influences the stability of the acid-producing fermentative bacteria and acetoclastic $CH_4$-producing archaea. It plays a major role in the oxidation-reduction potential of the anaerobic process. Thus, it directly impacts the metabolic pathway. In most of literature reports, a pH of 5.5 has been considered to be the optimum pH for $H_2$ production [3, 47, 70, 95]. The optimal initial pH range for the maximum $H_2$ yield or specific $H_2$ production rate is between pH 5.5 and 6.5 [95]. The optimal pH is highly dependent on the microorganism. The control of pH and alkalinity of a substrate is essential for first stage dark fermentation since organic acids produced tend to decrease the pH. The pH lower than 4.5 trends to inhibit the activity of hydrogenases. Low pH also causes in shift of metabolic pathways of dark fermentation microorganisms away from $H_2$ production. $H_2$-producing bacteria like *Clostridium acetobutylicum* can change metabolism from $H_2$ (acetate and butyrate pathway) to the production of solvents (acetone and butanol pathway) when the pH is decreased to less than 5.0. Alternatively, depending on the organism, low pH can shift the metabolism toward ethanol production [72]. Carbohydrate-based substrates provide good carbon and energy sources for $H_2$-producing bacteria. The fermentation process needs buffering of the growth medium, and to be supplemented with nutrients to enhance the growth of microorganisms and resist the pH change caused by organic acids produced [9, 55, 96]. $CH_4$ production is favored at alkaline pH exhibiting maximum activity at pH of 7.8–8.2 [97]. The rate of $CH_4$ production may decrease if the pH is lower than this optimal range. The pH is also an important factor for the stability of $CH_4$ production. The $H_2$ effluent which is rich in VFA, may cause a drop in pH if fed with high OLR. The pH adjustment can be achieved by an addition of alkali chemical, typically calcium carbonate or sodium hydroxide. A cheap material like ash was used to adjust the pH in an anaerobic reactor [98]. A stable $CH_4$ production process is characterized by the bicarbonate alkalinity in the range of 1000–5000 mg/L as $CaCO_3$. The ratio between VFA and alkalinity should be in the range of 0.1–0.25.

### 4.6. Temperature

Temperature is one of the most important factors affecting the growth of microorganisms. The operating temperature influences the growth rate of bacteria by influencing the biochemical reactions responsible for the maintenance of homeostasis and their metabolism. $H_2$-producing dark fermentation reactors can be operated in various temperature ranges from mesophilic (35–45°C), thermophilic (55–60°C) to extreme thermophilic (70–80°) conditions. Most of the $H_2$ dark fermentation studies have been conducted at temperature range of 35–45°C. Many mesophilic bacteria such as *Clostridium* sp. and *Enterobacter* sp. showed optimal $H_2$ production in the temperature range of 35–45°C [99]. A thermophilic $H_2$-producing bacterium gave higher $H_2$ yield compared to mesophilic bacteria [100]. When temperature rises, microbial growth rates increase due to the increase in the rates of chemical and enzymatic reactions in their cells. Thermophilic temperature makes the $H_2$ production process thermodynamically favorable with the $H_2$ yield of ~2.1 mol $H_2$/mol glucose, while mesophilic $H_2$ production gave the yield of ~1.7 mol $H_2$/mol glucose [101]. Although the $H_2$ yield from thermophilic temperature was slightly higher than that for mesophilic temperatures, the specific $H_2$ production rate (mmol $H_2$/h·gVSS) for thermophilic temperatures was 5–10 times higher than that from the mesophilic temperatures. Thermophilic $H_2$-producing bacteria has certain operation advantages such as low solubility of $H_2$ and $CO_2$, less influenced by the $H_2$ partial pressure, better solubility of the substrate, improved hydrolysis reaction as well as thermodynamic efficiency. Temperature is also a very important operation factor in the second stage for anaerobic digestion process. It determines the rate of anaerobic digestion process, particularly the rate of hydrolysis and methanogenesis. The thermophilic process could accelerate the biochemical reactions and give higher degradation efficiency as well as higher $CH_4$ production rates compared to mesophilic condition [102]. As temperature increases, the rate of retention time process is much faster and this results in more efficient operation and lowers the retention time requirement [97]. Thermophilic condition also increases in thermodynamic favorability of $CH_4$-producing reactions, decreases solubility of $CH_4$ and $CO_2$, and destruction of pathogens in the reactor effluent. Methanogens are extremely subtle to change in temperature and even a small temperature variation (2–3°C) can lead to VFA accumulation [103]. This decreases the $CH_4$ production rate for methanogens, especially at the thermophilic conditions. Maintaining the stable temperature is important for biohythane production.

### 4.7. Trace elements

Biohydrogen and biomethane production required various types of metal ions as micronutrients. These metal ions play a critical role in the metabolism of microorganisms. Metal ions such as $Fe^{2+}$, $Zn^{2+}$, $Ni^{2+}$, $Na^{+}$, $Mg^{2+}$, and $Co^{2+}$ play a pivotal role in both biohydrogen and biomethane process. Metals are essential to supplement in media for dark fermentation. These micronutrients might be required in trace amounts but they have an influential role as cofactors, transport processes facilitators, and structural skeletons of many enzymes (Fe-Fe hydrogenase and Ni-Fe hydrogenase) involved in the biochemistry of $H_2$ formation [104]. Therefore, several researchers have studied the effect of supplementation of Fe ion on biohydrogen production. For example, Lee et al. [105] studied the effect of Fe ion concentration (0–4000 mg/L) on $H_2$ fermentation and found that the $H_2$ production increased with iron concentration of 200 mg/L. The addition of Fe ion 200 mg/L influences the system positively with increasing $H_2$ production from 131 to 196 mL $H_2$/g sucrose. Ferchichi et al. [106] suggested that the supplementation with $Fe^{2+}$ ions (12 mg/l) led to a shift in their metabolic profile, for example, supplementation with $Fe^{2+}$ ion concentration of 12 mg/l caused a metabolic shift from lactic acid fermentation to butyric acid fermentation. Magnesium ions function as a cofactor of many enzymes such as kinases and synthetases. In glycolysis, many enzymes require magnesium ions as a cofactor. The activation of hexokinase, phosphofructokinases, glutaraldehyde-3-phosphate dehydrogenases, and enolases helps bacteria to metabolize substrate and produce energy component ATP [107]. Fe ion also plays a critical role in biomethane stage. The Fe ion is required by methanogenic archaea like *Methanosarcina barkeri* to synthesize protocheme via precorrin-2, which is formed from uroporphyrinogen III in two consecutive methylation reaction utilizing S-adenosyl-L-methionine [108]. Nickel is also an essential metal which plays a critical role in functioning of many enzymes that are responsible for $CH_4$ production such as monoxide dehydrogenase, hydrogenase, and methyl coenzyme M reductases.

## 5. Reactors configuration for biohythane production

The bioreactors in which the microorganisms are grown also play a crucial role. The design and the configuration of the fermenter help in the improvement of mixing characteristics and manipulation of overhead gas partial pressure. Parameters such as HRT and recycle ratio are influenced by the bioreactors configuration. The progress on two-stage system was presented based on the type of feeding substrates, classified as sugar-rich biomass, food/municipal waste, cellulose-based biomass, and palm oil mill effluent (POME). Over 20% of the publications reported so far focused on a system using sugar-rich synthetic wastewater. The most commonly used sugars were glucose and sucrose [10]. The maximum biohythane production was 3.21 mol $H_2$/mol hexose and 3.63 mol $CH_4$/mol hexose from glucose and acetic acid (synthetic wastewater) in CSTR reactor [109]. The summarized $H_2$ and $CH_4$ yield from various two-stage reactors configuration used for biohythane production is shown in **Table 4**. The schematic flow diagrams of each two-stage anaerobic fermentation systems for biohythane production are shown in **Figure 2**. The two-stage anaerobic fermentation is suitable for individual optimization of the $H_2$ and $CH_4$ production processes. For example, temperature-dependent process will be favored by the two-stage process, where high yield of $H_2$ could be achieved under thermophilic conditions, and stable maintaining of $CH_4$ production might be achieved under mesophilic conditions [13, 15, 21, 110]. Solubilization and saccharification of organic wastes with high solid content can be realized simultaneously during the first stage $H_2$ production [17, 74]. The two-stage anaerobic fermentation systems by integrated continuous stirred-tank reactor (CSTR) with anaerobic baffled reactor (ABR), CSTR with UASB, CSTR with CSTR, UASB with UASB, ASBR with UASB and stepped anaerobic baffled (SAB) were used for biohythane production (**Figure 2**.). The system with a CSTR and an upflow biofilter reactor for $H_2$ and $CH_4$ production from sucrose was established [89]. This system inoculated with heat-treated sludge as inoculum achieved a maximum $H_2$ yield of 1.62 mol $H_2$/mol hexose. The second stage reactor inoculated with raw anaerobic sludge achieved a maximum $CH_4$ yield of 323 L $CH_4$/kg COD. The analysis of COD balance showed that 13.5% of the influent COD was transformed to $H_2$ and 70% of the influent COD was transformed to $CH_4$. A CSTR $H_2$ and CSTR $CH_4$ system fed with synthetic glucose medium using the same anaerobic sludge as inoculums was reported [18]. By optimizing the inoculums-to-substrate ratio (2:1) in this CSTR-CSTR system, the $H_2$ yield and the methane yield increased to 2.75 and 2.13 mol/mol hexose, respectively, with 10 g/L glucose as a substrate, which corresponded to a total energy recovery of 82%. A similar reactor configuration was also used by Lee et al. [25] and Hafez et al. [109]. A synthesis wastewater containing glucose and acetic acid produced 2.6 mol $H_2$/mol hexose and 426 mL $CH_4$/kg COD via continuous fermentation in CSTR [109]. The stable $H_2$ production in the CSTR was possibly due to the introduction of a gravity settler after the $H_2$ CSTR for $H_2$-producer retention. A complete CSTR system for $H_2$ and $CH_4$ production from cassava stillage was developed [12]. The gas yields under thermophilic conditions with high

| Reactors ($H_2$ and $CH_4$) | Feedstock and conditions | $H_2$ production yield (L-$H_2$/kg VS) | $CH_4$ production yield (L-$CH_4$/kg VS) | Biogas composition | References |
|---|---|---|---|---|---|
| CSTR and CSTR | Olive pulp, temperature of 35 and 35°C, pH of 5 and 7 | 190 | 160 | 1.6% $H_2$<br>38.3% $CO_2$<br>60% $CH_4$ | [110] |
| UASB and UASB | Desugared molasses, temperature of 70 and 55°C, pH of 5 and 7 | 89 | 307 | 16.5% $H_2$<br>38.7% $CO_2$<br>44.8% $CH_4$ | [11] |
| CSTR and UASB | Sugarcane syrup, temperature of 37 and 30 °C, pH of 5.5 and 7.5 | 88 | 271 | 19.6% $H_2$<br>62.6% $CO_2$<br>10.9% $CH_4$ | [111] |
| ASBR and UASB | POME, temperature of 55 and 35°C, pH of 5.5 and 7.5 | 210 | 315 | 14% $H_2$<br>32% $CO_2$<br>51% $CH_4$ | [13] |
| CSTR and UASB | POME, temperature of 55 and 35 °C, pH of 5.5 and 7.5 | 135 | 414 | 13.3% $H_2$<br>32.2% $CO_2$<br>54.4% $CH_4$ | [15] |
| CSTR and CSTR | Biowaste, temperature of 55 and 35 °C, pH of 5.5 and 8 | 41 | 102 | 6.7% $H_2$<br>40.1% $CO_2$<br>52.3% $CH_4$ | [112] |
| CSTR and UASB with gas upgrade systems | Wheat straw, temperature of 70 and 37°C, pH of 6.9 and 7.5 | 270 | 179 | 46–57% $H_2$<br>0.4% $CO_2$<br>43–54% $CH_4$ | [113] |
| CSTR and ABR | Food waste, temperature of 55 and 35°C, pH of 5.5 and 7.5 | 205 | 464 | 15% $H_2$<br>54.5% $CO_2$<br>30.5% $CH_4$ | [21] |
| SAB | Petrochemical wastewater, temperature of 21 and 21°C, pH of 5.5 and 7.5 | 88 | 318 | 16% $H_2$<br>27% $CO_2$<br>52% $CH_4$ | [114] |

**Table 4.** Hydrogen and methane yield from various reactor configurations used for two-stage biohythane production.

organic loading (13 g COD/L·d) were 56.6 L $H_2$/kg TS, and 249 L $CH_4$/kg volatile solid (VS), respectively. Chu et al. [21] developed a two-stage thermophilic CSTR reactor and a mesophilic ABR reactor with the heat-treated digested sludge to recirculation to first reactor for $H_2$ and $CH_4$ production from organic fraction of municipal solid wastes (OFMSW). The separation of $H_2$ and $CH_4$ production was successful by operating the $H_2$ reactor at a controlled HRT of 1.3 days, and pH of 5.5. Kongjan et al. [11] established a biohythane process from wheat straw hydrolysate by two-stage extreme thermophilic UASB and thermophilic UASB. Specific

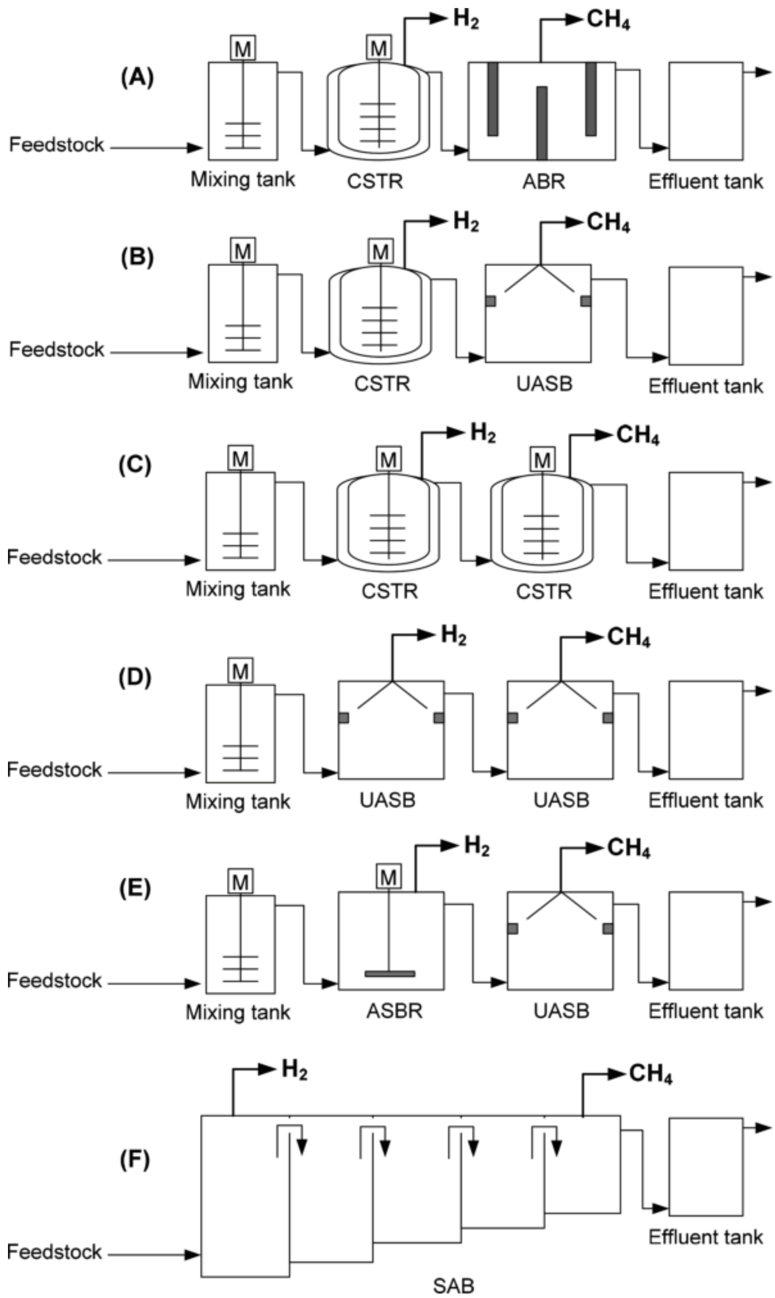


**Figure 2.** Schematic flow diagrams of two-stage anaerobic fermentation systems for biohythane production by integrated CSTR with ABR (A), CSTR with UASB (B), CSTR with CSTR (C), UASB with UASB (D), ASBR with UASB (E) and SAB (F).

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$H_2$ and $CH_4$ yields of 89 mL-$H_2$/g-VS (190 mL $H_2$/g sugars) and 307 mL $CH_4$/gVS, respectively were achieved simultaneously with the overall VS removal efficiency of 81% by operating with total HRT of 4 days. A biohythane gas with the composition of 16.5% $H_2$, 44.8% $CH_4$, and 38.7% $CO_2$ could be produced at high production rates (3.5 L/L·d). *Thermoanaerobacter wiegelii*, *Caldanaerobacter subteraneus*, and *Caloramator fervidus* were responsible for $H_2$ production in the $H_2$-UASB reactor. Meanwhile, the $CH_4$-UASB reactor was dominated with methanogens of *Methanosarcina mazei* and *Methanothermobacter defluvii*. Successful biohythane production from palm oil mill effluent (POME) by two-stage thermophilic ASBR followed by mesophilic UASB was achieved by Mamimin et al. [13]. The continuous biohythane production rate of 4.4 L/L·d with biogas composition of 14% $H_2$, 51% $CH_4$ and 35% $CO_2$ was achieved. O-Thong et al. [15] established two-stage thermophilic CSTR and mesophilic UASB with methanogenic effluent recirculation to $H_2$ reactor for biohythane production from POME. The 30% recirculation rate of methanogenic effluent could keep pH at optimal pH with two times increase in $H_2$ production when compared with non-recirculation systems. The $H_2$ and $CH_4$ yields were 135mL $H_2$/gVS and 414 mL $CH_4$/gVS, respectively. Biohythane gas composition was composed with 13.3% $H_2$, 54.4% $CH_4$, and 32.2% $CO_2$. *Thermoanaerobacterium* sp. was dominated during $H_2$ production from POME, whereas archaea belonging to *Methanosarcina* sp. and *Methanoculleus* sp. were dominated in the $CH_4$ reactor. A two-stage process with methanogenic effluent recirculation flavored *Thermoanaerobacterium* sp. in the $H_2$ reactor and efficiently for energy recovery from POME. Elreedy et al. [114] established biohythane production from petrochemical wastewater containing mono-ethylene glycol by a novel stepped anaerobic baffled (SAB) reactor. The reactor was continuously operated for 5 months at constant hydraulic retention time (HRT) of 72 h with hydrogen and methane yield of 88 mL $H_2$/gVS and 318 mL $CH_4$/gVS, respectively.

Reactors are considered to be practical and economical for industrial $H_2$ production, particularly via mixed culture fermentation [70, 100]. The two main bioreactor configurations: suspended and attached, or immobilized, growth types have been applied to optimize fermentation process for $H_2$ production through advancements in active biomass concentration and substrate conversion efficiency [101, 115]. Most studies on $H_2$ production from carbohydrate rich substrates have been conducted in suspended CSTRs, which are simple to construct, easy to regulate both acidity and temperature, and give complete homogeneous mixing for direct contact between the substrate and active biomass [1, 70, 72]. Furthermore, the CSTR is very suitable for substrates with a high-suspended solid (SS) content, typically with a volatile solid (VS) content of 2–12% [48]. However, in CSTR reactor, HRTs must be greater than the specific growth rate of the microorganisms in order to control the proper concentration of microbial biomass, but faster dilution rates risk active biomass washout [1, 67] leading to process failure. In addition, cell density retained in CSTR is limited, since the active biomass has the same retention time as HRT, resulting in process instability caused by the fluctuation of environmental parameters, including acidity and then having the consequence of limiting substrate degradation and $H_2$ production. To overcome the above mention problem, a new configuration of a continuous flow reactor is required to decouple the cell mass retention from HRT and subsequently retain higher cell densities in the reactor, such as UASB and ASBR, which can be achieved through granules and biofilm [47, 91, 115, 116]. Cells immobilization can be employed successfully by using a diluted waste stream with relatively small reactor volumes in ASBR, SAB, and UASB reactors. However, such a reactor configuration has a poor mass transfer system, which is mainly caused by a lack of mixing; this can lead to gases accumulating in the biofilm or granular sludge that risk losing $H_2$ by $H_2$-consuming bacteria [92, 101]. Mass transfer can be improved by mechanical stirring or liquid recirculation, depending on the reactor type and configuration. Also, applying proper bioreactor shapes and optimizing reactor dimensions such as the height to diameter ratio can help to improve mass transfer efficiency [91, 98, 117–119].

The anaerobic conversion of VFA to $CH_4$ is mainly associated with sequential stages of acetogenesis and methanogesis. When optimizing a methanogenic process using VFA rich, soluble organic matters, the goal is to maximize both $CH_4$ production and VFA degradation, while keeping the reactor stable [37]. The acetogenesis is limited mainly by VFA degradation, especially propionate that is the rate-limiting factor in the second stage anaerobic process. The investigation into optimizing the methanogenic reactor is mostly carried out by varying OLRs via increasing the substrate concentration or decreasing the HRTs to obtain satisfactory performance [25, 120]. The main signs of methanogenic reactor instability or overloading are decrease in pH [121]. As a drop of pH actually corresponds to VFA accumulation, pH below 6.3 has an impact on enzyme activity in the microorganisms involved in the second stage anaerobic digestion. Methanogenic archaea can function properly in a pH range between 6.5 and 7.8 [122]. Thus, a buffering solution is needed in order to resist a pH drop from VFA accumulation in the methanogenic process and maintain stability. The main buffer in the anaerobic digester is bicarbonate ($HCO_3$), which is usually added to carbohydrate rich substrates before feeding them to the first stage of $H_2$ fermentation because the first stage needs to be controlled with pH within the favorable range of 5–6 for $H_2$-producing bacteria [123, 124]. Lee et al. [25] found that the pH drop below 6.4 caused by the accumulation of 122 mM VFA in the attached growth reactor operated at 55°C and fed with 11.0 gVS/L·d (5.13 d HRT) of the food waste fermentation. The pH could inhibit the bioactivity of methanogenesis. Meanwhile, the maximum $CH_4$ production rate of 2100 mL $CH_4$/L·d with a $CH_4$ content of 65% was obtained at pH around 7.5, where the reactor was operated at a 7.7 day HRT (7.9 gVS/L·d OLR) and almost VFA degradation was achieved. For the high rate anaerobic reactor, UASB reactor was operated at double OLR comparing to CSTR at thermophilic temperature (55°C) which providing better VFAs degradation than mesophilic temperature (35°C) [125]. This is mainly attributed to the increase of chemical and biological reaction rates for operating temperature of thermophilic condition and the organic acid oxidation reactions become more energetic at higher temperature [126, 127]. Because the $H_2$ reactor effluents are in soluble form of organic matters as the consequence of hydrolysis and acidogenesis in the first stage, the reactor type used to convert these soluble organic matters to $CH_4$ in the second stage are based on high rate biofilm systems as reviewed by Demirel et al. [27]. Cell mass is retained well in the biofilm/granular aggregates in biofilm systems, leading to have much higher sludge retention time (SRT) compared to HRT, which provides the advantage that the reactor can run at a higher flow rate and can tolerate higher toxic concentrations [128]. Various types of high rate biofilm systems such as UASB, ABR, and SAB can be operated by continuous feeding with the $H_2$ reactor effluent, with HRTs of less than 5 days [114, 125, 129, 130]. Among the high rate reactor types, the UASB is the most popular for anaerobic treatment of soluble organic matters due to the large surface area of granular sludge, which provides fast biofilm development and improves methanogenesis. Also clogging and channeling occur less in the UASB reactor than other biofilm systems [121].

## 6. Application of biohythane process

Methane is being commonly used, not only in the chemical industry but also in transport as compressed natural gas (CNG), which has been regarded as the clean energy carrier in comparison to gasoline or diesel. By combining the advantages of $H_2$ and $CH_4$, biohythane is considered one of the important fuels involved in achieving the transition of technical models from a fossil fuel-based society to renewable-based society. $CH_4$ used as a fuel for vehicle has weak points on its narrow range of flammability, slow burning speed, poor combustion efficiency as well as requirement for high ignition temperature of CNG-powered vehicles. Interestingly, $H_2$ perfectly complements the weak points of $CH_4$ such as the hydrogen/carbon ratio which is increased by adding $H_2$, which reduces greenhouse gas emissions. Adding $H_2$, thus, improves the fuel efficiency and can extend the narrow range of flammability of $CH_4$. The flame speed of $CH_4$ can be greatly increased by adding $H_2$, eventually reducing combustion duration and improving heat efficiency. The quenching distance of $CH_4$ can be reduced by the addition of $H_2$, making the engine easy to ignite with less input energy. A two-stage process technique, combining acidogenesis and methanogesis appears to give more efficient waste treatment and energy recovery than a single methanogenic process [13]. As the results reported by Kongjan and Angelidaki [129], mixed gas of $CH_4$, $CO_2$, and $H_2$ with the volumetric content of 44.8, 38.7, and 16.5%, respectively, containing approx. 10% $H_2$ on energy basis could be achieved. This specification was found to be most suitable for burning directly in the internal combustion engines [131] and could be biohythane. In addition to economical concern, the two-stage thermophilic anaerobic process has been previously evaluated that the payback time is around 2–6 years, depending on the disposal costs of organic wastes/residues [28].

Various types of organic wastes can be used as substrate for biohythane production such as starch wastewater, palm oil mill effluent (POME), biowaste, sugarcane syrup, olive pulp, desugared molasses, food waste, and organic solid waste [13, 18, 19]. $H_2$ and $CH_4$ yield from two-stage biohythane production of palm oil mill effluent (POME) was 201 mL $H_2$/gCOD and 315 mL $CH_4$/gCOD, respectively [13], which were higher than those of starch wastewater (130 mL $H_2$/gCOD and 230 mL $CH_4$/gCOD, respectively) [18], sugarcane syrup (88 mL $H_2$/gCOD and 271 mL $CH_4$/gCOD, respectively) [111], and biowaste (21 mL $H_2$/gCOD and 55 mL $CH_4$/gCOD, respectively) [112]. $H_2$ and $CH_4$ yield from two-stage biohythane production of olive pulp (190 mL $H_2$/gVS and 160 mL $CH_4$/gVS, respectively) [110] was lower than that of food waste (205 mL $H_2$/gVS and 464 mL $CH_4$/gVS, respectively) [21]. Successful biohythane production from POME by two-stage thermophilic $H_2$ reactor and mesophilic $CH_4$ reactor was achieved with biohythane production rate of 4.4 L/L·d with biogas composition of 51% $CH_4$, 14% $H_2$, and 35% $CO_2$ [13]. POME is a suitable substrate for $H_2$ production in terms of high biogas production volume. Energy analysis of two-stage anaerobic fermentation process has greater net energy recovery than the single stage $H_2$ production and single stage $CH_4$ production process. O-Thong et al. [15] applied two-stage thermophilic fermentation and mesophilic methanogenic process with methanogenic effluent recirculation to $H_2$ reactor for biohythane production from POME. The pH two-stage reactor was control by recirculation of methanogenic effluent with $H_2$ and $CH_4$ yield of 135 mL $H_2$/gVS and 414 mL $CH_4$/gVS, respectively. Flow diagram of successful thermophilic two-stage anaerobic fermentation for biohythane from POME at lab scale 5 L CSTR and 25 L UASB, semi-pilot scale 50 L CSTR and 250 L UASB and industrial scale 5 $m^3$ CSTR and 25 $m^3$ UASB are shown in **Figure 3**.

Improvement methods such as effluent recirculation to mix with feedstock in $H_2$ reactor, biomethane gas recirculation to $H_2$ reactor, and the combined effluent recirculation to $H_2$ reactor with biomethane gas sparging to $CH_4$ reactor were reported to enhance biohythane production (**Figure 4**). The two-stage anaerobic fermentation process with methanogenic sludge recirculation (two-stage recirculation process) could be successfully operated and maintained at pH around 5.5 in $H_2$ reactor without any alkaline addition [21]. The recirculation of part of the methanogenic sludge to a H2 reactor was provided as the buffer for the first stage. Kim et al. [132] also reported the recycling of a methanogenic effluent to a $H_2$ reactor with $H_2$ production increased from 1.19 to 1.76 $m^3$ $H_2$/$m^3$·d, and decreased the requirement for alkali addition. $H_2$ yield from the two-stage anaerobic fermentation with the recirculation process was 2.5–2.8 mol/mol hexose [25], which was relatively high comparing to 4 mol/mol hexose from the maximum theoretical $H_2$ yield. The recirculation of the $CH_4$ effluent to hydrogen reactor could protect the $H_2$ fermentation process from a sharp drop in pH or organic overloading. Operations with the circulation of heat-treated sludge performed considerably better than those with the recirculation of raw sludge with respect to both the $H_2$ production rate and yield [19]. Lee et al. [25] improved two-stage anaerobic fermentation for biohythane production by biomethane gas sparging to second stage and recirculation biomethane effluent for pH adjustment in $H_2$ reactor. The gas yields were 2.3 mol $H_2$/mol hexose and 287 L $CH_4$/kg COD, respectively, while TS of food waste was kept at 10%. The recirculation of methanogenesis effluent provides ammonia-rich buffer, which flavors $H_2$-producing bacteria eventually and improves the performance of the $H_2$ reactor. Liu et al. [34] were the first group to develop a two-stage CSTR-CSTR system for mesophilic $H_2$ and $CH_4$ production using household solid waste as both inoculum and substrate. The yields of $H_2$ and $CH_4$ were 43 and 500 L/kg VS, respectively, while the TS of the $H_2$ CSTR was maintained at 10%. $CH_4$ production was over 20% higher than that in single-stage $CH_4$ fermentation. Cavinato et al. [120] established a two-stage CSTR-CSTR reactor under thermophilic condition for biohythane production from municipal solid waste. The $H_2$ and $CH_4$ gas yields were 52 L $H_2$/kg VS and 410 L $CH_4$/kg VS, respectively. Willquist et al. [113] proposed a biohythane process from wheat straw including pretreatment, $H_2$ production using *Caldicellulosiruptor saccharolyticus*, $CH_4$ production using a methanogenic consortium, and gas upgrading using an amine solution. The first reactor was extreme thermophilic CSTR and the second reactor was mesophilic UASB applying for biohythane production. A biohythane gas with the composition of 46–57% $H_2$, 43–54% $CH_4$, and 0.4% $CO_2$ could be produced at high production rates (2.8–6.1 L/L·d), with 93% chemical oxygen demand (COD) reduction, and a net energy yield of 7.4–7.7 kJ/g dry straw. The $CO_2$

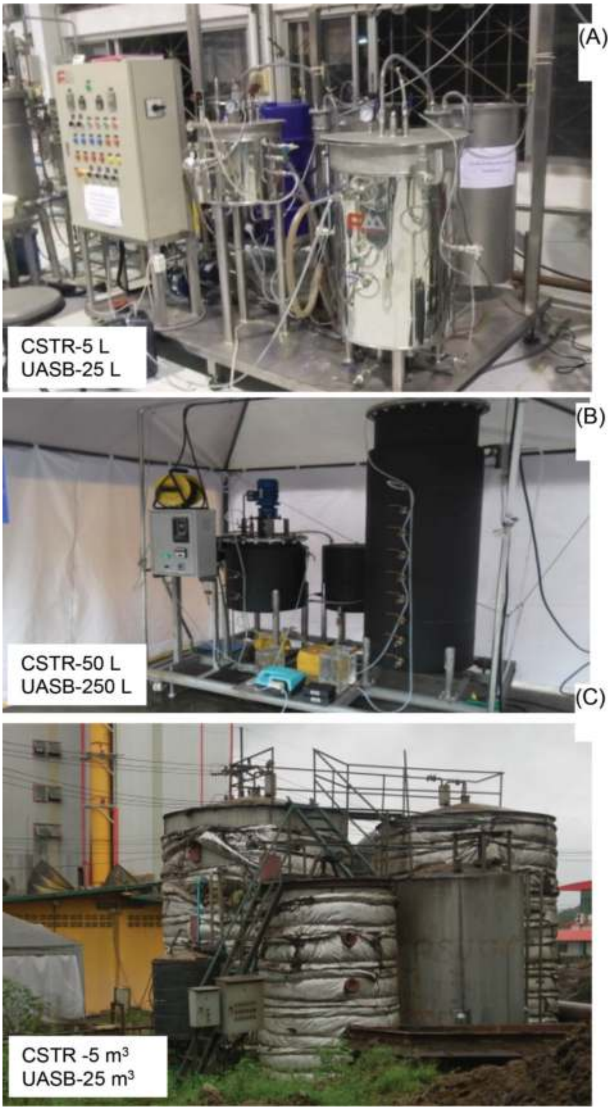


**Figure 3.** Flow diagram of scaling-up of the two-stage anaerobic fermentation for biohythane production from POME; a lab scale 5 L CSTR and 25 L UASB (A), semi-pilot scale 50 L CSTR and 250 L UASB (B), industrial scale 5 $m^3$ CSTR and 25 $m^3$ UASB (C).

has to be removed before the biogas can be used as hythane by an amine solution, consisting of a mixture of 40% N-methyldiethanolamine (MDEA), 10% piperazine (PZ) and 50% water, by weight. This is a solvent commonly used in industry for the removal of $CO_2$ in various mixtures of gases, including biogas.

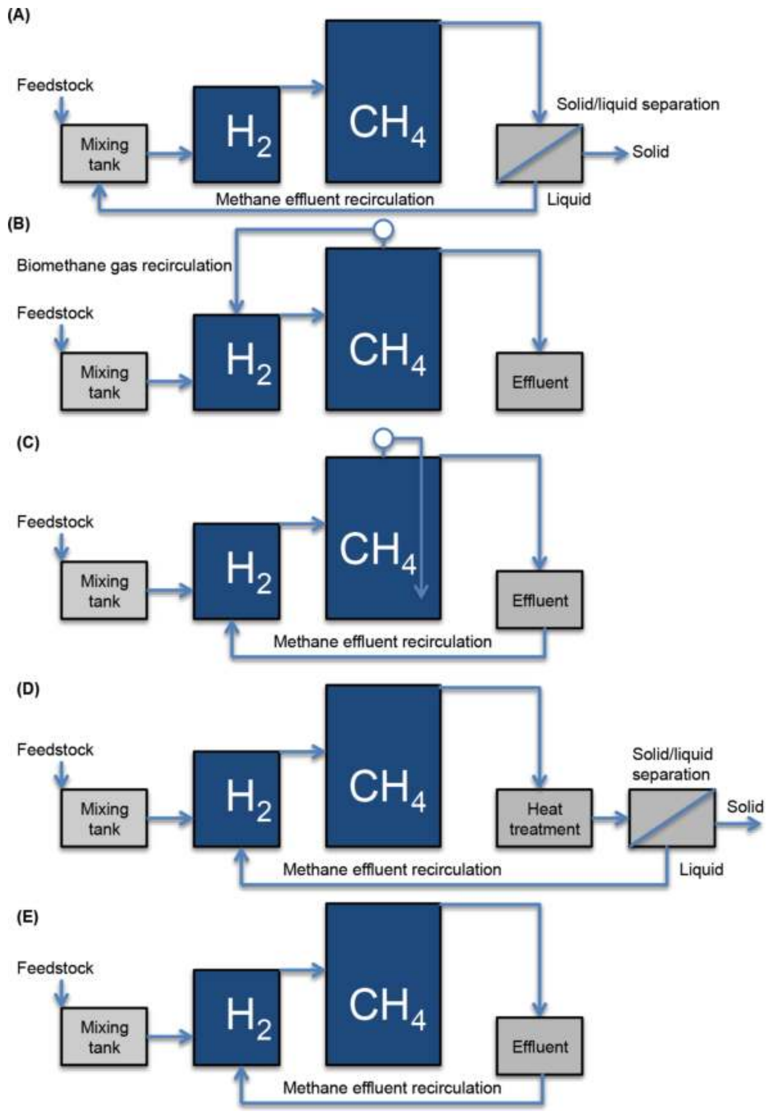


**Figure 4.** Schematic flow diagrams of gas yield improving for two-stage anaerobic fermentation for biohythane production by liquid methane effluent recirculation method (A), biomethane gas recirculation method (B), the combine liquid methane effluent recirculation and biomethane mixing method (C), liquid methane effluent heated recirculation method (D), and mixed solid and liquid methane effluent recirculation (E).

## 7. Conclusions

Biohythane via two-stage anaerobic fermentation using organic waste could be a promising technology for higher energy recovery and a cleaner transport biofuel than the biogas.

The $H_2/CH_4$ ratio of range 0.1–0.25 is suggested for biohythane. A flexible and controllable $H_2/CH_4$ ratio afforded by two-stage fermentation is of great importance in making biohythane. Biohythane can be achieved by two-stage anaerobic fermentation; in the first stage, organic wastes is fermented to $H_2$, $CO_2$, VFA, lactic acid and alcohols. Effluents from first stage containing VFA, lactic acid, and alcohols are converted to $CH_4$ in the second stage by methanogens under a neutral pH range of 7–8 and HRT of 10–15 days. The pH of 5–6 and an HRT of 2–3 days are optimized for first stage that flavor acidogenic bacteria to convert organic wastes to $H_2$. *Clostridium* sp., *Enterobacter* sp., *Caldicellulosiruptor* sp., *Thermotoga* sp., and *Thermoanaerobacterium* sp., are efficient $H_2$ producers in the first stage. *Methanosarcina* sp. and *Methanoculleus* sp. played an important role in the second stage $CH_4$ production. The combination of biohydrogen and biomethane production from organic wastes via two-stage anaerobic fermentation could yield a gas with a composition like hythane (10–15% of $H_2$, 50–55% of $CH_4$, and 30–40% of $CO_2$) called biohythane. Biohythane could be upgraded to bio-based hythane by removing $CO_2$. The two-stage anaerobic fermentation could increase COD degradation efficiency, increase net energy balance, increase $CH_4$ production rates as well as high yield and purity of the products. In addition, the two-stage process has advantages of improving negative impacts of inhibitive compounds in feedstock, increased reactor stability with better control of the acid production, higher organic loading rates operation, and significantly reducing the fermentation time.

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