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Review

Critical Review on Two-Stage Anaerobic Digestion with H_2 and CH_4 Production from Various Wastes

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Abstract: Anaerobic digestion (AD) is a promising method for resource recovery from various wastes. Compared to the conventional single-stage AD process, a two-stage AD process with separate H_2 and CH_4 production provides higher energy recovery efficiency and enhanced operation stability. The stage separation makes it possible to apply optimal conditions for different functional microorganisms in their respective stages. This review elaborates the mechanisms of the two-stage AD process and evaluates recent research trends on this topic. A comprehensive comparison between single- and two-stage AD processes is made from the perspective of biogas production, organics degradation, energy recovery, and operation stability. The main influence factors on the two-stage AD process are discussed, including substrates, inoculum, and operation parameters, such as pH, temperature, etc. Upgrading technologies for the two-stage AD process are assessed. The microbial communities in the two-stage AD process for treating different substrates and the influence factors on microbial systems are also summarized. Furthermore, future research opportunities for enhancing the application of this technology are highlighted.

Keywords: two-stage anaerobic digestion; hydrogen; methane

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1. Introduction

Anaerobic digestion (AD) is an efficient and environment-friendly method for treating wastewater, sewage sludge, and organic wastes. During AD, complex organic compounds are converted to CH_4 and CO_2. Both the biogas, which is regarded as an alternative clean fuel to fossil fuels, and the digestate, which can be further utilized as fertilizer, can enhance the recycling of waste materials. Applications of the AD process, including the conventional single-stage AD, two-stage AD, and the combination of AD and other processes, have been explored in many studies. The two-stage AD process, usually with the production of H_2 in the first dark fermentation (DF) stage and CH_4 in the second AD stage, has been shown to be a promising and attractive technology. Previous studies showed that stage separation appeared to be effective for the enhanced degradation of biomass and better operation stability [1]. Mixed biohythane, which contains both H_2 and CH_4 generated from stage-separated AD, is also believed to be more competitive than sole CH_4, the major gaseous product of single-stage AD [2]. Figure 1 shows the growing interest on two-stage AD in recent years and, particularly, how biohythane production has attracted increasing attention during the past ten years (Figure 2).

In general, the production of biogas and organic removal through the use of two-stage AD could be improved significantly by modifying certain aspects of the process, such as utilization of suitable substrates and inoculum, application of digestate recirculation, etc. In addition, by optimizing the pH, temperature, organic loading rate (OLR), hydraulic retention time (HRT), and other vital parameters, the performance of two-stage AD could also be enhanced to a certain degree. The primary objective of this review is to summarize recent research concerning the two-stage AD process and the effect of the influence parameters and upgrading technologies on the process. Moreover, a comparison between the single- and two-stage AD processes from various perspectives is specially discussed in this review.

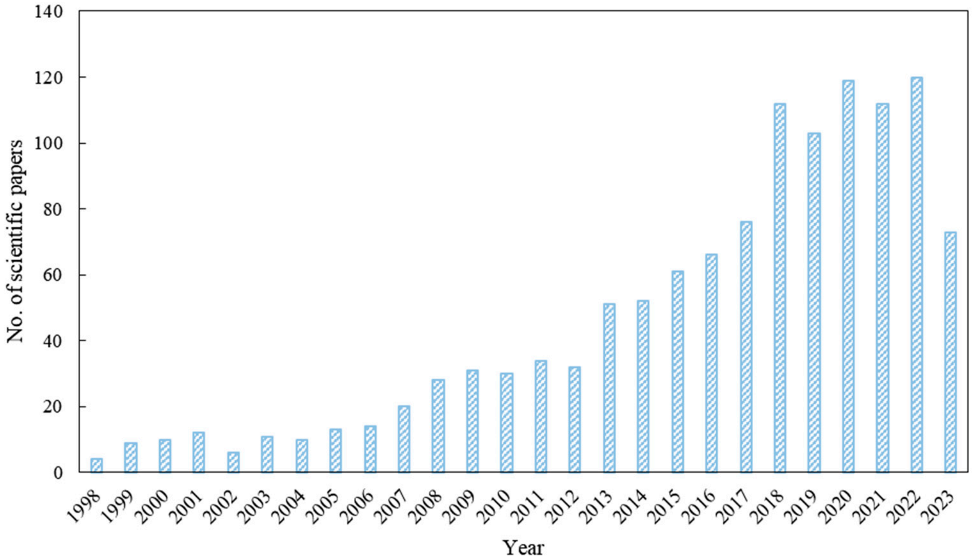

Figure 1. The number of scientific papers on the investigation of two-stage AD in 1998–2023.

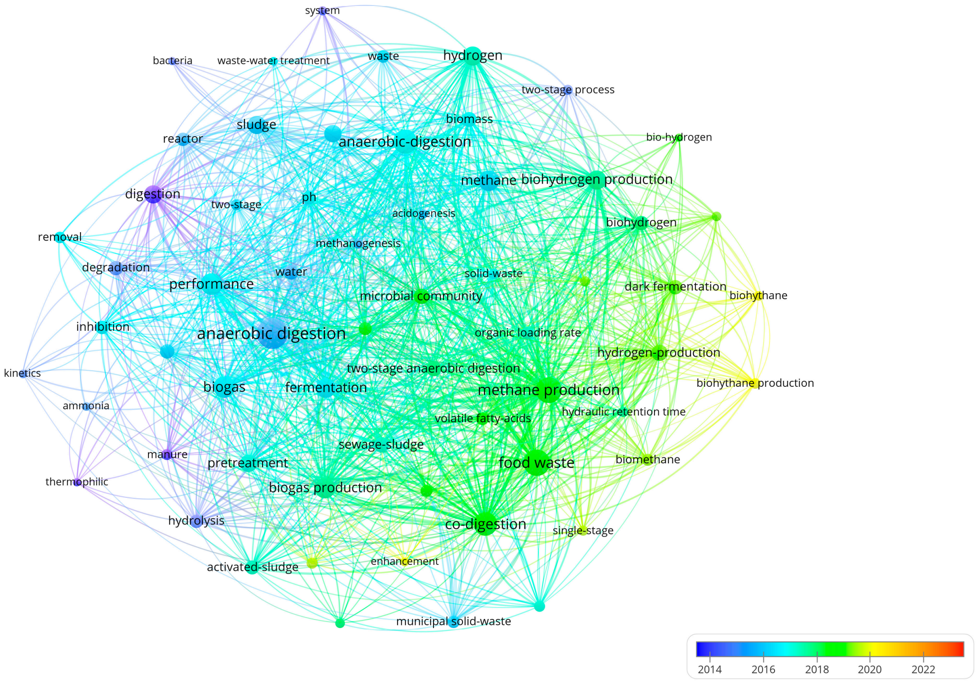

Figure 2. The research trends of key topics in two-stage AD investigation in 2014–2023.

2. Comparison of Single- and Two-Stage AD Processes

During the AD process, the conversion of organic compounds is conducted by a variety of microorganisms which participate in the following four steps:

- Hydrolysis, in which organic macromolecules, such as carbohydrates, proteins, lipids, and other large polymers, are degraded into small monomers by hydrolytic and fermentative bacterial consortia;

- Acidogenesis, in which the monomers are further degraded into various metabolic products, mainly consisting of volatile fatty acids (VFAs), alcohols, lactate, NH_4^+, H_2, and CO_2 by acidogenic bacteria;
- Acetogenesis, in which products from acidogenesis are converted to H_2, CO_2, and acetate by acetogenic bacteria;
- Methanogenesis, in which CH_4 is produced from CO_2, acetate, or methyl compounds by methanogens.

As shown in Figure 3, microbes related to all the four steps are commonly combined in one reactor to establish a synergic microbial consortium for the metabolism of organics in the conventional single-stage AD process [3]. The hydrolysis and/or methanogenesis steps are likely to be rate-limiting steps during the whole process depending on the type of substrate [3]. However, the relevant microbes for the four steps require different operating conditions and it is a significant challenge to manage the balance of the complex microbial community.

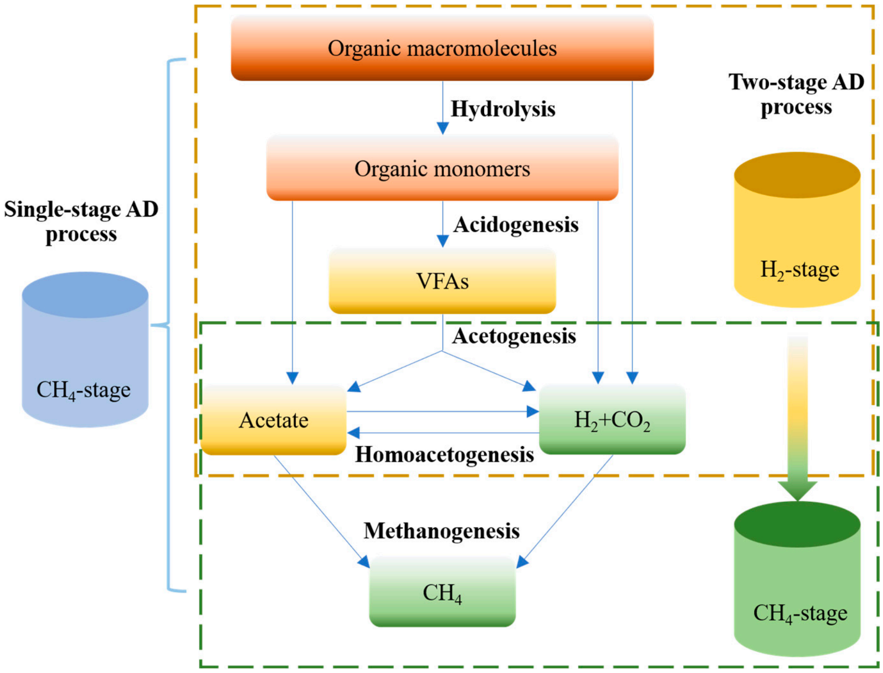

Figure 3. Single- and two-stage AD processes.

In the two-stage AD system comprising H_2 and subsequent CH_4 production, the methanogenesis step is physically restricted to occur in the second stage [4,5]. The organics are degraded to H_2, CO_2, and organic acids in the former stage and are further transformed to CH_4 in the latter stage. Therefore, different microbes can be enhanced at their optimum conditions in different stages and thus show better performance. For instance, methanogens have a less thick cellular membrane and are more sensitive to inhibitors than fermentative bacteria and thus require more stable operation conditions [4]. Moreover, previous study using thermodynamics analysis of the Gibbs free energy during H_2 and CH_4 production indicated faster growth rates of microbes in H_2 fermentation [6]. CH_4 production, in contrast, required longer HRT for stable degradation of substrates [7]. Moreover, some researchers have considered the former stage as a pretreatment process to increase hydrolysis of hardly biodegradable substrates, which is used to ensure higher CH_4 production in the ensuing stage [8].

A comparison of operation performance in single- and two-stage AD processes for treating wastewater, sewage sludge, and organic wastes based on data from previous studies is shown in Table 1. Theoretically, the two-stage system may achieve higher energy recovery but needs more energy loading for heating another reactor [6]. Evaluation of the energy balance and economic analysis are yet to be conducted.

Table 1. (a) Comparison of the operation performance in single- and two- stage AD processes treating wastewater. (b) Comparison of the operation performance in single- and two- stage AD processes treating sewage sludge and organic wastes [1].

(a)

Substrate	**Single-Stage Process**				**Two-Stage System**						**Reference**
					H_2 Stage		**CH_4 Stage**		**The System**		
	CH_4 Yield (mL/g-COD_{added})	**Reactor Type and Key Variables**	**COD Removal (%)**	**Energy Recovery (as Biogas, kJ/g-COD_{added})**	**H_2 Yield (mL/g-COD_{added})**	**Reactor Type and Key Variables**	**CH_4 Yield (mL/g-COD_{added})**	**Reactor Type and Key Variables**	**COD Removal (%)**	**Energy Recovery (as Biogas, kJ/g-COD_{added})**	
Synthetic wastewater	227.43	Packed bed reactor (PBR); 37 °C; HRT = 12 h	74.0	8.15	61.81	PBR; 37 °C; HRT = 12 h	263.58	PBR; 37 °C; HRT = 12 h	97.3	10.09	[9]
Synthetic wastewater	275.25	Upflow anaerobic sludge blanket (UASB); 37 °C; HRT = 12 h	95.4	9.85	157.45	UASB; 37 °C; HRT = 12 h	234.43	UASB; 37 °C; HRT = 12 h	97.8	10.07	[9]
Paper mill effluent (PME)	213.5	Batch; Initial pH = 7; 37 °C	58.87	7.66 [2]	31.8	Batch; Initial pH = 5; 37 °C	>400	Batch; Initial pH = 7; 37 °C	78.05	Not available (N/A)	[10]
Palm oil mill effluent (POME)	227	UASB; 35 °C; HRT = 17 d	84	9.08	210	Anaerobic sequencing batch reactor (ASBR); 55 °C; HRT = 2 d	315	UASB; 28–34 °C; HRT = 15 d	H_2 stage: 38 CH_4-stage: 95	15.34	[11]
Cheese whey	312.1	Sequencing batch reactor (SBR); 55°C; HRT = 12.5 d	N/A	11.1 [2]	7.3	Semi-continuously-fed reactor; 35 °C; HRT = 1.5 d	340.4	SBR; 55 °C; HRT = 12.5 d	N/A	12.1 [2]	[12]
Starchy wastewater	111.8–134.8	Batch; 40 °C	N/A	5.0	92.0	Batch; 55 °C	138.0	Batch; 40 °C	N/A	8.2	[13]
Agricultural wastewater	203.7–245.6	Batch; 40 °C	N/A	6.1–7.3	N/A	Batch; 55 °C	114.1	Batch; 40 °C	N/A	2.1–6.2	[13]
Dairy wastewater	260.8–325.3	Batch; 40 °C	N/A	9.4–11.7	5.7	Batch; 55 °C	227.6–294.9	Batch; 40 °C	N/A	10.5–11.1	[13]
Sugary wastewater	160.8–182.9	Batch; 40 °C	N/A	6.2–6.7	N/A	Batch; 55 °C	82.8–107.3	Batch; 40 °C	N/A	2.8–3.3	[13]

Table 1. *Cont.*

(b)

Substrate	Single-Stage Process				Two-Stage System						Reference
					H_2 Stage		CH_4 Stage		The System		
	CH_4 Yield (mL/g-VS_{added})	**Reactor Type and Key Variables**	**VS Removal (%)**	**Energy Recovery (as Biogas, kJ/g-VS_{added})**	**H_2 Yield (mL/g-VS_{added})**	**Reactor Type and Key Variables**	**CH_4 Yield (mL/g-VS_{added})**	**Reactor Type and Key Variables**	**VS Removal (%)**	**Energy Recovery (as Biogas, kJ/g-VS_{added})**	
Sewage sludge	105.2	Batch; 35 °C	N/A	3.76	38.8	Batch; 35 °C	96.9	Batch; 35 °C	N/A	3.88	[14]
Waste activated sludge (WAS)	101.2	Continuous stirred tank reactor (CSTR); 37 °C; HRT = 15 d	N/A	4.02	74.5	CSTR; 55 °C; HRT = 5 d	150.7	CSTR; 37 °C; HRT = 10 d	N/A	7.00	[15]
					6.7	CSTR; 37 °C; HRT = 5 d	127.8	CSTR; 37 °C; HRT = 10 d	N/A	5.16	[15]
Activated sludge + food waste (FW)	N/A	CSTR; 37 °C; HRT ≈ 17 d	61.0	N/A	N/A	CSTR; 37 °C; HRT ≈ 12 d	N/A	CSTR; 37 °C; HRT = 3 d	71.5	N/A	[16]
WAS + OFMSW [3]	490	CSTR; 55 °C; HRT = 20 d	86.92	17.59 [2]	24	CSTR; 55 °C; HRT = 3 d	570	CSTR; 55 °C; HRT = 17 d	87.69	20.72 [2]	[17]
Sewage sludge + wine vinasse	120.20	CSTR; 35 °C; HRT = 5 d	38.32	4.32 [2]	22.90	CSTR; 55 °C; HRT = 1 d	212.56	CSTR; 35 °C; HRT = 4 d	53.19	7.88 [2]	[18]
WAS + FW	79.9	Reactor; 37 °C; HRT = 12 d	44.6	2.9	64.5	Reactor; 55 °C; HRT = 1.1 d	154.1	Reactor; 37 °C; HRT = 6 d	54.3	6.2	[19]
Sewage sludge + rice straw	171	Batch; 55 °C	43.8	5.5	21	Batch; 55 °C	266	Batch; 55 °C	60.4	8.8	[20]
Fruit and vegetable waste + WAS + olive mill wastewater + cattle manure	340	ASBR; 37 °C; HRT = 20 d	73.6	12.7	79.4	CSTR; HRT = 5.33 d	463	ASBR; HRT = 8 d	90.9	13.44	[21]
					79.4	CSTR; HRT = 5.33 d	730	ASBR; HRT = 8 d; biogas from H_2 stage recirculated to CH_4 stage	91.15	21.06	[21]

Notes: [1] The colors of the table represent performance in single-stage (blue) and two-stage AD processes (salmon), respectively. [2] The values were calculated based on the biogas yield. The lower heating values of H_2 and CH_4 are 10.8 kJ/L and 35.9 kJ/L, respectively [13]. [3] OFMSW-organic fraction of municipal solid waste.

2.1. Biogas Production

As one of the intermediate metabolites during the AD process, H_2 could be rapidly utilized by hydrogenotrophic methanogens or other H_2-consuming microbes. CH_4 is thus assumed to be the main component of the biogas products in the single-stage process, apart from the by-product CO_2. In two-stage systems, however, both H_2 and CH_4 could be recovered in separate stages under different operating conditions. H_2, a well-known clean and sustainable fuel, has thus attracted wide attention in many studies of two-stage AD processes. The H_2 stage could also be a CO_2-stripping step to enrich the CH_4 content in the biogas generated from the CH_4 stage, promising lower potential costs of biogas upgradation compared to the single-stage process [16,22]. The biogas from the two-stage AD process could also be captured as a whole (i.e., biohythane [23]) and could be developed as a green and efficient vehicle fuel after removing the CO_2 [6]. The two-stage AD system could generally provide more competitive biogas products compared to single-stage processes in those cases.

In most cases, the two-stage AD process has not shown obvious promotion of CH_4 yield on wastewater (Table 1a). The comparison of COD removal efficiency and energy yield indicated that two-stage AD might still show a relative advantage considering the whole system due to the previous recovery of H_2 in the H_2 stage [9,24]. However, considering the higher investment cost of the two-stage AD system, single-stage AD might be superior for wastewater treatment [25]. Solid wastes, especially those with a large quantity of complex and hardly biodegradable components, were more treatable using two-stage AD (Table 1b). With proper operation conditions, the CH_4 yield of solid wastes in two-stage AD could be double that from the single-stage process [21]. Furthermore, a certain amount of H_2 could also be obtained in the two-stage AD treatment of solid wastes [15]. The key factor responsible for the difference between wastewater and solid wastes is the biodegradable degree of the substrate. The single-stage AD process could be an effective and economical choice when treating easily biodegradable substrates. However, when treating hardly biodegradable substrates, the two-stage AD process was more advantageous with respect to biohythane production.

2.2. Degradation of Organics

The volatile solid (VS) or COD removal efficiencies of two-stage AD systems for both liquid and solid wastes were found to be higher than those of single-stage processes in most cases (Table 1). Moreover, two-stage processes were demonstrated to be more tolerant of higher organic loading. For instance, the single-stage AD of fruit waste (banana peel) showed a slight advantage for CH_4 and energy yields when the total solid (TS) of the feedstock was 1.5%, while the two-stage AD system showed 4.1 and 4.8 times higher values for CH_4 and energy yields, respectively, when TS was up to 2.0% [8]. In another investigation treating a mixture of waste activated sludge and food waste, comparable CH_4 production and removal efficiency at an OLR of 1.2 g-VS/L/d were obtained from both single- and two-stage AD processes [19]. However, the digestion was inhibited with a higher OLR of 4.5 g-VS/L/d in the single-stage process, while it was still stable and highly efficient at an OLR as high as 6.1 g-VS/L/d in the two-stage system [19].

2.3. Energy Recovery

The energy recovery of two-stage AD systems included the bioenergy of both the H_2 and CH_4 produced in separate stages. The two-stage AD could also achieve higher energy recovery than single-stage systems in most cases (Table 1). For example, the overall energy recovery efficiencies of the two-stage AD were 28% and 74% higher than the single-stage process under mesophilic (37 °C) and thermophilic (55 °C) conditions in the H_2 stage, respectively [15].

2.4. Operation Stability

Two-stage AD processes were usually more stable than single-stage processes as the operation parameters (pH, OLR, etc.) were more conveniently optimized via the separation of H_2 and CH_4 production and the pretreating effect of the H_2 stage. The ratio of total VFA and alkalinity (TVFA/ALK) has been used as an indicator of AD process stability, with a range below 0.4 suggesting steady mesophilic methanogenic performance and above 0.8 indicating a failure in operation [8]. Liu et al. reported that the average TVFA/ALK was 0.53 at a high OLR of 4.5 g-VS/L/d in a single-stage process, while it was below 0.4 in the two-stage system, corresponding to the performance reported in Section 2.2 of [19].

Two-stage AD also showed higher tolerance to inhibitors than single-stage processes. For example, the two-stage system (7.3–29.1% inhibited) performed better than single-stage processes (22.8–39.0% inhibited) when spiked with cefazolin [26].

3. Influence Factors of the Two-Stage AD Process

The quantity and quality of biogas generated from the two-stage AD process differed according to the type of substrate and inoculum, pH, temperature, OLR, HRT, nutrients, and inhibitors, etc., as discussed below.

3.1. Substrate

Both sole-substrates and co-substrates have been applied in two-stage AD systems. The performance of the systems varied based on the components and pretreatment methods of the substrates.

3.1.1. Sole Substrates

Synthetic or actual wastewater (sugarcane syrup, molasses, cassava wastewater, cheese whey, etc.), which contained monosaccharides (glucose, xylose, etc.), disaccharides (lactose, sucrose, etc.), polysaccharides (e.g., starch), VFAs, or other easily biodegradable organics, were commonly used substrates in the two-stage AD process. Using lipids as a substrate, the system showed higher theoretical biogas yields than when using carbohydrates and proteins [3,27], but lipid-rich wastewater was not as capable of producing H_2 as carbohydrate-rich wastewater, though proper pretreatment methods could improve its degradation [28]. Palm oil mill effluent (POME) is a kind of wastewater containing lipids, carbohydrates, and VFAs, and thus was much more easily degraded than other lipid-rich wastewaters [29–31].

The two-stage AD process when treating solid wastes showed more complex and diverse features than when treating wastewater. For example, a two-stage system with thermophilic (55 °C) alkaline fermentation followed by a mesophilic (37 °C) AD process was demonstrated to be capable of reducing the diversity and total abundance of bacterial pathogens in sewage sludge [15]. Lignocellulosic-rich solid wastes usually contain a high content of cellulose, hemicellulose, and lignin. The structural carbohydrates (e.g., cellulose) in the cell walls would be much more difficult to degrade compared to the non-structural carbohydrates (e.g., starch). Therefore, almost all of those substrates were cut and/or milled and most were pretreated to derive monosaccharides, such as glucose, before AD [14]. Food waste contains large amounts of organics and is generally considered a suitable substrate for AD processes. Researchers have paid much attention to food waste in the past few years (Figure 2). However, studies on using the two-stage AD process to treat protein-rich substrates are quite limited due to the low H_2 yield of protein. This could be attributed to the limitation of the theoretical H_2 yield, as well as the H_2 consumption during the degradation of some amino acids [3,27]. Some studies have applied pretreatment methods to deal with food waste containing certain amounts of hardly biodegradable components. For instance, alkali, acid, and H_2O_2 pretreatments all enhanced the performance of the two-stage AD treatment of bean wastes, among which H_2O_2 pretreatment was found to be the most effective method, with a biogas yield of 152.1 mL H_2/g-VS and 462.6 mL CH_4/g-VS [32].

3.1.2. Co-Substrates

For substrates rich in easily biodegradable carbohydrates, the accumulation of VFAs and lack of nitrogen and essential trace elements might be key adverse factors for the AD process [33]. On the other hand, substrates with a low C/N ratio or with hardly biodegradable components might also lead to difficulties in the AD process due to the lack of available organic compounds. The supplementation of pure carbon, nitrogen sources, or mineral salts would increase operation costs, while co-digestion of different kinds of substrates could be a more economical choice [28]. Furthermore, co-digestion might synergistically improve biogas production and energy conversion efficiency and maintain better process stability by adjusting the C/N ratio, supplementing nutrients and trace elements, creating a synergistic effect on microbes and diluting possible toxic substances [34,35]. Therefore, co-digestion has also attracted much attention recently (Figure 2).

A comparison between sole- and co-substrates in two-stage AD processes is shown in Table 2. Several organic solid wastes rich in proteins and minerals were found to be quite efficient in co-digestion with distillery effluent, which contains a high carbohydrate load, achieving as high as 2.73 and 2.56 times H_2 and CH_4 production compared to use of sole distillery effluent [33]. Protein was found to be a necessary nitrogen source for anaerobic microorganisms to maintain metabolism in the AD process [14]. The theoretical nitrogen requirement for the AD process (COD:N:P = 100:1:0.2) might even become higher with increasing temperature (COD:N:P = 100:6:0.5 at 55 °C) due to the high growth rate of thermophiles [36]. Moreover, the protein could be hydrolyzed into low-molecular-weight peptides and amino acids, which could be further fermented into VFAs and NH_4^+ [14]. Lignocellulosic-rich substrates could also be acceptable supplementary carbon sources for substrates with a low C/N ratio, especially when suitable pretreatment methods are applied. For example, the co-digestion of NaOH-pretreated cornstalk and sewage sludge achieved H_2 and CH_4 yields of 13.4 mL/g-VS and 172.6 mL/g-VS, respectively, which were around 13 and 1.4 times those obtained from sole sewage sludge digestion [37].

Table 2. Comparison of operation performance between sole and co-substrates in the two-stage AD process [1].

Sole Substrate-a			**Sole Substrate-b**			**Co-Substrates**		**Reference**
Substrate	**H_2 Yield (mL/g-VS_{added})**	**CH_4 Yield (mL/g-VS_{added})**	**Substrate**	**H_2 Yield (mL/g-VS_{added})**	**CH_4 Yield (mL/g-VS_{added})**	**H_2 Yield (mL/g-VS_{added})**	**CH_4 Yield (mL/g-VS_{added})**	
Chlorella (Ch)	36.40	166.18	Molasses	N/A	N/A	90.12	319.52	[38]
			Glycerol waste	N/A	N/A	39.80	577.33	
			POME	N/A	N/A	53.70	545.35	
			Napier grass	N/A	N/A	39.98	401.69	
			Empty fruit bunch	N/A	N/A	22.02	466.35	
Empty fruit bunch	35.3	236.9	POME	53.1	259.1	16.6	348.6	[34]
Decanter cake	35.9	251.2				50.9	247.8	
Palm press fiber	39.6	279.1				60.9	247.5	
Oil palm frond	66	202.7				23.4	299.5	
Oil palm trunk	28.1	216.4				18.3	364.3	
Groundnut deoiled cake	N/A	N/A	Distillery effluent	55.13 [2]	24.95 [2]	150.7 [2]	64.06 [2]	[33]
Mustard deoiled cake	N/A	N/A				109.1 [2]	43.13 [2]	
Distillers' dried grain with solubles	N/A	N/A				144.2 [2]	63.84 [2]	
Algal biomass	N/A	N/A				116.1 [2]	38.88 [2]	
FW	47.37	498.06	Glyceryl trioleate	0.60	520.37	23.98	555.89	[28]
FW	149.3	270.9	Sewage sludge	17.9	70.6	174.6	264.1	[39]
FW	29.38	N/A	Pulp & paper sludge	2.294	N/A	64.48	432.3	[40]
Sewage sludge	1.0	122.1	Cornstalk	N/A	N/A	13.4	172.6	[37]

Notes: [1] All the studies listed in this table used batch reactors. The colors of the table represent performance of sole substrate-a (blue), sole substrate-b (salmon) and co-substrates (green), respectively. [2] Calculated as mmol/L.

Substrates with a low C/N ratio (such as sewage sludge and manure) were usually co-digested with substrates that had a high C/N ratio (such as food waste) in the two-stage AD process [16,41,42]. The optimum C/N ratio for each kind of substrate varied and thus different ratios of the co-substrates were also considered [40,43–45]. With proper pretreatment, such as by ozonation, both H_2 and CH_4 production of the co-substrates could be promoted [28]. Heat, aeration and filtration pretreatment were all found to be effective in eliminating methanogenic activity of the H_2 stage and promoting biogas yields from a mixture of food waste and sewage sludge [46]. However, NaOH or H_2SO_4 pretreatment seemed to be only suitable for enhancing H_2 production [47].

3.2. Inoculum

Inoculum was considered to be one of the crucial factors in the two-stage AD process. The two stages were dominated by different metabolic processes demanding different types of microbes. Therefore, the inocula of the two stages also differed.

3.2.1. Inoculum of the H_2 Stage

Although some substrates, such as sewage sludge, could be used as the inoculum for H_2 production, external inocula were applied in most cases. Pure bacterial cultures chosen for specific substrates were believed to be related to higher H_2 yields [48]. However, the situation could be different when the CH_4 stage was also considered. A pure culture (*Clostridium butyricum* TISTR1032) resulted in a higher H_2 production potential of sugarcane juice but a lower CH_4 yield in the CH_4 stage compared to mixed cultures (granules from a UASB reactor), and thus led to a lower overall energy yield [49].

Mixed microbes were generally chosen for the H_2 stages due to their better adaptability to complex environmental conditions [7]. However, the mixed microbes often introduced H_2 consumers to the H_2 stage, such as hydrogenotrophic methanogens, homoacetogens, etc., and thus decreased the H_2 yield [50]. Therefore, the inoculum was usually pretreated to enrich the H_2-producing bacteria and to inhibit the activity of H_2 consumers. Thermal pretreatment was the most commonly utilized method [51]. Rafieenia et al. discovered that thermal pretreatment could inhibit the methanogens in the inoculum of the H_2 stage when treating food waste, while alkali and aeration pretreatment showed limited domestication of the inoculum [52]. However, a significantly higher H_2 yield was not achieved when thermal treatment was applied to the inoculum compared to that without treatment, which might be a result of the substrate (steam-exploded cornstalk) containing fermentation inhibitors that selectively favored H_2 fermentation [53].

The form of inoculum also had an influence on the operation of the reactor. In batch experiments, it was reported that the H_2 production potential of sugarcane juice with immobilized cells of the pure culture was 1.2-fold higher than that of free cells, while no significant difference was observed in H_2 production potential with inoculation with UASB granules in granule and suspended form [49]. However, with suspended cells applied in a semi-continuous process, the substrate flow rate increased with decreasing HRT and thus cells would be washed out, which might ultimately lead to reactor failure [54]. A cell immobilization method was found to be effective to solve the problem [55]. A bacterial immobilization method was found to be effective to recover biogas production from low-strength wastewater in a two-stage UASB system [56].

3.2.2. Inoculum of the CH_4 Stage

Most two-stage AD systems used mixed microbes as inocula in the CH_4 stage. In contrast to the inoculum for H_2 production, pretreatment methods were seldom applied to the inoculum of the CH_4 stage.

3.3. pH

The microbial communities were highly influenced by pH, and the optimum pH differed in the two stages. Except for a few studies adopting an alkaline condition [15], the pH value in the H_2 stage was in the range 5.0–6.5 in most cases [57]. The optimal pH differed with different substrates. For a mixture of expired food products and hydrolysate from used disposable nappies, H_2 production was favored with increasing pH from 5.5 to 6.0 [35]. With respect to dried maize silage, the H_2 production rate increased by 46%, while the H_2 content decreased by 17% with increasing pH from 5.0 to 5.5 [58]. It was necessary to control the proper pH range with an alkaline substance for most of the H_2-reactors due to the accumulation of VFAs that accompanied H_2 production. The results from different pH-controlling approaches showed that alkali salt (as $NaHCO_3$) performed better on the H_2 yield of cheese whey than alkali (as NaOH) [59]. Alkaline wastes could also be used as pH-control substitutes to reduce the cost of commercial alkalis and enable reuse of wastes [60].

In the batch tests, suitable initial pH in the H_2 stage differed over a larger range. An acid initial condition was effective for some of the substrates, such as acid-hydrothermal pretreated *Chlorella* sp. biomass and steam-explosion pretreated sugarcane bagasse, both of which showed the best H_2 production at an initial pH of 6.0 [13,61]. However, a neutral or weakly alkaline initial pH was also used in many studies. Neutral or alkaline initial pH (7.0 and 9.0, respectively) in batch tests treating OFMSW could shorten the lag phase of H_2 production compared with an acid initial condition (pH = 5.5) [7]. Despite different initial pH sets, pH would decrease to the range 5.0–6.5 if there was no pH adjustment during the operation [7,43].

The pH in the CH_4 stage was usually within the range 6.5–8.0 [62]. With the initial pH ranging from 6.0 to 8.0 in batch tests treating *Chlorella* sp. biomass, the highest CH_4 yield was obtained at an initial pH of 7.5 [13].

3.4. Temperature

Reports showed that thermophilic (50–55 °C) and mesophilic (30–40 °C) temperatures were generally applied for H_2 and CH_4 production, respectively. More specifically, 55 °C and 37 °C proved to be the optimum values [10,43]. For the H_2 stage, the thermophilic (55 °C) condition was found to be more efficient for both H_2 and subsequent CH_4 production, as well as for the removal of pathogens, than the mesophilic (37 °C) condition when treating sewage sludge [15]. For the CH_4 stage, CH_4 production under a mesophilic (35 °C) condition was better than that under a thermophilic (55 °C) condition in batch tests treating low-strength wastewater from a beverage factory [56]. However, opposite trends were found for OFMSW and POME [63,64]. The elevated temperatures might enhance the hydrolysis of hardly biodegradable substrates but result in poorer operation stability.

3.5. OLR

The OLR was an essential parameter in AD process. Insufficient biomass supply at a low OLR could not provide sufficient feedstock for biogas production, whereas overfeeding substrates at a high OLR could lead to the accumulation of VFAs, which, in turn, led to a pH drop and suppressed the metabolisms of microorganisms. Insufficient time for hydrolysis at a high OLR also influenced the degradation and removal efficiency of organics. Optimum OLRs were determined for different kinds of substrates. A maximum biogas conversion efficiency of 71.06% was achieved from molasses wastewater at an OLR of 30 g-COD/L/d [65]. Increasing the OLR from 6 to 15 g-VS/L/d enhanced the hydrolysis of food waste in the H_2 stage but had no significant impact on the specific methane yields in the CH_4 stage [22].

3.6. HRT

With decrement in HRT, the OLR increased and provided adequate organics for biodegradation conducted by the microorganisms. Therefore, both H_2 and CH_4 yields were inversely related to increment in the HRT of the two stages [66–68]. However, the methanogenesis step could be inhibited when the HRT was too short, due to the accumulation of

VFAs at a correspondingly high OLR. Suitably prolonged HRT enabled better degradation of the substrates [69] and lower risk of microbial washout for both stages [41,67,70].

3.7. Nutrients

Nutrients, such as C, N, P sources as well as essential trace elements, were added in many two-stage AD systems. These nutrients played a vital role in the growth and stability of the microbial communities.

The C/N ratio was a key factor for the stable operation of the AD process. Chen et al. found that the H_2 yield increased at first and then decreased with an increasing C/N ratio from 7.2 to 40.1 [44]. The C/N ratio could be controlled by the addition of a carbon or nitrogen source. It was found that NH_4Cl showed better promotion of H_2 production from low-strength wastewater compared to urea, while its effect on CH_4 production did not show much difference [56]. NH_4HCO_3 was also used as a nitrogen source and could maintain appropriate pH with the formation of a buffer system of $CO_3{}^{2-}/HCO_3{}^{-}$ for the digestion of cornstalk [71].

Trace metals, such as Fe, Ni, Co, etc., were essential co-factors of key enzymes involved in the AD process. They could affect microbial activities and consequently promote biogas production and process stability within an appropriate range of dosage [31,61,72].

3.8. Inhibitors

The presence of some toxic pollutants, such as antibiotics [26], chloroform [43], tetrabromobisphenol A [73], etc., could impact negatively on two-stage AD processes. Two-stage AD systems showed more tolerance to these toxic pollutants compared to the single-stage AD process [26]. Co-digestion could be a method to reduce the inhibition of toxic pollutants [43].

Sulfur was found to an important element in amino acids. However, it could also reduce biogas quality and show toxicity to various microbial groups in the AD process when degraded to H_2S or other sulfur compounds [74]. Two-stage AD systems showed a capacity for simultaneous biogas production and sulfur removal [75,76].

4. Upgrading Technology

4.1. Type of Reactors

Most studies of two-stage AD processes were conducted at laboratory scale using batch or semi-continuous reactors. A few investigations were carried out in pilot [77,78] or full-scale [79] systems. The type of reactors treating solid wastes were mainly CSTRs, while those for wastewater treatment showed more variety. Compared to the UASB reactors, the two-stage packed bed reactor (PBR) system was superior to single-stage PBR when treating synthetic wastewater in terms of biogas production and organic removal efficiency [9]. In another study treating the mixture of food waste and sewage sludge, the effluent from the H_2 stage was fed to the subsequent CH_4 stage, during which the anaerobic sequencing batch reactor (ASBR) achieved better total CH_4 conversion efficiency (69.9%) than the UASB reactor (56.9%), while higher CH_4 production rates (1.96 L/L/d) were attained in the UASB reactor compared to the ASBR (1.10 L/L/d) [80].

4.2. Integrating Technology

Integrated approaches using a two-stage AD process with other technologies, such as microbial fuel cells (MFCs), photo fermentation (PF), etc., have attracted much attention due to more diversified products and better elimination of organic wastes [81,82].

Research on MFCs in integrating systems has focused on their potential for the simultaneous degradation of residual organics and electricity energy recovery [82,83]. A coupling system of a two-stage AD process and subsequent MFC achieved higher energy conversion efficiency and overall COD removal (60.0% and 94.1%, respectively) from water hyacinth, compared to the two-stage AD (19.0% and 81.5%, respectively) [84]. The coupling system also achieved a higher COD removal efficiency of 98.4% compared to 73.2% for the two-stage system when treating the liquid fraction of pressed municipal solid waste [24].

In contrast to DF H_2 production, which was usually used in the first stage of a two-stage AD process, PF is a biohydrogen production method requiring adequate light. It could further convert by-products of DF to H_2 and has been applied between DF and AD. A DF-PF-AD system improved H_2 production and thus achieved higher total energy yield compared with a two-stage AD process [81].

Bioethanol was considered to be a sustainable energy carrier and the integration of ethanol fermentation and a subsequent two-stage biohythane system assured an efficient utilization of biowaste [85]. A coupled bioethanol and biohythane production system promoted substrate degradation (91.3%), carbon conversion efficiency (75.1%), and energy recovery (22,156.6 kJ/kg-$COD_{removed}$) [86].

4.3. Digestate Recirculation

Digestate recirculation in two-stage AD systems usually referred to the circulation of liquid effluent from the CH_4 stage to the H_2 stage with a certain ratio [45,78]. The recirculation could help to maintain the optimal pH range for the H_2-producing bacteria (HPB) [66] and compensate for alkalinity and the nitrogen source required by the H_2 stage, resulting in reduced operational costs [29,87]. However, the recirculation might have an inhibiting effect on H_2 production due to the H_2-consuming activities of non-HPB and thus inactivation of circulated sludge [88]. Moreover, high concentration of ammonia would inhibit HPB. Considering possible inhibition, a suitable recirculation rate was a key factor in the two-stage system with digestate recirculation [29,87].

4.4. Biogas Recirculation

Biogas recirculation has attracted much attention in recent years, reusing CO_2 and/or H_2 produced in the AD process to enhance biogas production [89].

Sparging biogas from the CH_4 stage, which mainly consisted of CO_2 and CH_4, could reduce the H_2 partial pressure in the H_2 stage and thus improve the H_2 yield [90–93]. On the other hand, it was observed that injection of CO_2 into the H_2 stage of a pilot-scale two-stage system increased VFA production [94], which might lead to pH drop and unstable H_2 production.

Recirculating biogas from the H_2 stage to the CH_4 stage could benefit CH_4 production, as H_2 and CO_2 are vital substrates for the methanogenic process [95]. The maximum specific CH_4 yield (0.73 L/g-VS) generated from co-digestion of several organic wastes was achieved in this regard at a high OLR of 4.0 g-VS/L/d, resulting in a 56% higher energy recovery compared to the two-stage system without biogas recirculation [21]. However, the proportion of CH_4 in the biogas might decrease due to the dilution effect of the recirculated CO_2 [95].

5. Microbial Communities

The efficiency of the two-stage AD process is strongly associated with the abundance and activities of specific anaerobic microbes. The dominant bacterial and archaeal communities in the two-stage AD process are shown in Table 3.

Table 3. Dominant microorganisms in the two-stage AD process.

Substrate	Biogas Yield (mL/g-COD_{added})	Dominant Bacteria in H_2 Stage	Dominant Bacteria in CH_4 Stage	Dominant Archaea in CH_4 Stage	Reference
Synthetic wastewater	H_2: 61.81–157.45 CH_4: 263.58–234.43	*Spirochaetaceae* *Bacteroidaceae* *Clostridiaceae* *Erysipelotrichaceae*	*Spirochaetaceae* *Synergistaceae* *Thermotogaceae* *Porphyromonadaceae*	*Methanosaetaceae*	[9]
POME	H_2: 210 CH_4: 310	*Thermoanaerobacterium acidoterolans*, *Thermoanaerobacterium thermosaccharolyticum*, *Thermococalles* sp., *Clostridium acetobutylicum*	N/A	*Methanobrevibacter* sp. *Methanosarcina* sp. *Methanoculeus* sp. *Sulfolobus* sp. *Aeropyrum* sp.	[96]
POME	H_2: 53.1 ^{1} CH_4: 259.1 ^{1}	*Clostridium* sp.	N/A	*Methanocorpusculum* sp. *Thermococcus* sp.	[34]
POME	H_2: 60–188 CH_4: 200–345	*Clostridium* sp., *Enterococcus* sp., *Marinomonas* sp., *Thermoanaerobacterium* sp.	*Clostridium* sp. *Fervidobacterium* sp. *Ruminococcus* sp.	*Methanosarcina* sp. *Methanoculleus* sp.	[29]
Sewage sludge	H_2: N/A CH_4: N/A	*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Chloroflexi*, *Nitrospira*, *Actinobacteria*	N/A	N/A	[97]
Waste activated sludge	H_2: 6.7–74.5 ^{1} CH_4: 127.8–150.7 ^{1}	*Actinobacteria* *Nitrospirae* *Proteobacteria*	*Actinobacteria* *Proteobacteria*	*Methanoculleus*	[15]
POME + various kinds of solid wastes	H_2: 16.6–60.9 ^{1} CH_4: 247.5–364.3 ^{1}	*Enterobacter* sp. *Clostridium* sp. *Megasphaera* sp.	N/A	*Methanocorpusculum* sp. *Methanomicrobium* sp. *Thermococcus* sp. *Methanosphaera* sp.	[34]

Note: ^{1} Based on VS_{added}.

5.1. Bacterial Communities

The functional bacteria classified as phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were widely detected in AD processes. The phylum *Firmicutes* participated in the hydrolysis, acidogenesis, and acetogenesis steps of a wide range of substrates [62,98]. The phylum *Bacteroidetes*, which can hydrolyze carbohydrates or proteins and transform organic substances to produce VFAs, was considered to participate in the hydrolysis and acidogenesis steps [62]. The phylum *Proteobacteria* includes important acetogenesis bacteria which could utilize glucose and various kinds of VFAs [9]. Moreover, a special group of bacteria, homoacetogens, could convert H_2 and CO_2 to acetate and thus act as competitors against H_2-consuming methanogens.

5.2. Archaeal Communities

Methanogenic archaea were the main functional microbes in the methanogenesis step and occurred mostly in the CH_4 reactor. CH_4 was produced by complex methanogenesis pathways, such as hydrogenotrophic, aceticlastic, methylotrophic, and methyl reduction pathways, among which the former two occurred most commonly [99].

The hydrogenotrophic methanogenesis (HM) pathway, which is also called the CO_2 reduction pathway, was the most common pathway utilized by nearly all methanogens [100]. The CO_2 was reduced during the HM pathway with H_2 as the primary electron donor and thus could be affected by homoacetogens that competed for H_2 [101].

The aceticlastic methanogenesis (AM) pathway was related to the dismutation of acetate to CO_2 and CH_4 [101]. The AM pathway was observed to occur only in the families *Methanosaetaceae* and *Methanosarcinaceae* [99]. Nearly two-thirds of the CH_4 produced in nature are believed to come from the methyl group of acetate [100]. Apart from the AM pathway, the acetate could also be degraded to CH_4 by the cooperation of syntrophic acetate oxidation bacteria (such as species in the genus *Clostridium*), that can oxidize acetate to H_2 and CO_2, and HM methanogens [101].

5.3. Influence Factors on Microbial System

The microbial communities in the two-stage AD process usually differed from those of the single-stage process [9]. The type of substrates exerted an influence on the microbial communities in two-stage AD systems, as shown in Table 3. Both the bacterial and the archaeal diversity in the co-digestion systems of POME and several solid wastes were higher than in the single-stage systems [34]. The pretreatment methods of the substrates could also influence the microbial communities in AD processes [102]. Furthermore, the type of inocula also affected the microbial communities [103].

Previous studies found that the microbial diversity and abundance of microbial communities in two-stage AD processes were closely associated with the digestion conditions. For instance, the relative abundances of *Firmicutes* and *Actinobacteria* increased with mixing intensities ranging from 30 rpm to 120 rpm in the H_2 stage [98]. Temperature affected both the chemical reactions and biological activities in AD processes. Thermophilic conditions usually led to lower microbial community diversities [45,104,105]. Compared with relatively stable archaeal communities, the bacterial communities were much more sensitive to variation in HRT/OLR [67,104]. Digestate recirculation simultaneously improved bacterial diversity and alleviated over-acidization in the H_2 stage [105].

6. Outlook

The two-stage AD process has been demonstrated to be a promising technology for producing biomethane and biohythane from various organic substrates. However, some technical challenges still need to be addressed before application of this technology. Firstly, mixed substrates with a suitable mixing ratio should be explored to enlarge the application of the two-stage AD process. The advantages of two-stage over single-stage AD when treating hardly biodegradable substrates should be fully considered. Secondly, mechanisms underpinning the interaction of substrates, microorganism, and environmental factors should be studied to further improve the operation efficiency of the two-stage AD process. In particular, research about how the archaeal community is affected by operation parameters is still inadequate. Comparison between single-stage and two-stage AD processes in this regard needs further study. Lastly, the optimization of reactor design and scaling up of this technology should be further investigated for practical application of this process in the future.

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