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Review

Two-Stage Anaerobic Digestion for Green Energy Production: A Review

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Abstract: Anaerobic digestion (AD) is a biotechnological process in which the microorganisms degrade complex organic matter to simpler components under anaerobic conditions to produce biogas and fertilizer. This process has many environmental benefits, such as green energy production, organic waste treatment, environmental protection, and greenhouse gas emissions reduction. It has long been known that the two main species (acidogenic bacteria and methanogenic archaea) in the community of microorganisms in AD differ in many aspects, and the optimal conditions for their growth and development are different. Therefore, if AD is performed in a single bioreactor (single-phase process), the optimal conditions are selected taking into account the slow-growing methanogens at the expense of fast-growing acidogens, affecting the efficiency of the whole process. This has led to the development of two-stage AD (TSAD) in recent years, where the processes are divided into a cascade of two separate bioreactors (BRs). It is known that such division of the processes into two consecutive BRs leads to significantly higher energy yields for the two-phase system (H_2 + CH_4) compared to the traditional single-stage CH_4 production process. This review presents the state of the art, advantages and disadvantages, and some perspectives (based on more than 210 references from 2002 to 2024 and our own studies), including all aspects of TSAD—different parameters' influences, types of bioreactors, microbiology, mathematical modeling, automatic control, and energetical considerations on TSAD processes.

Keywords: two-stage anaerobic digestion; hydrogen; methane; mathematical models

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1. Introduction

Anaerobic digestion of organic wastes has become a very attractive biotechnology in recent years, mainly in the fields of renewable energy sources and biofuels. This biotechnology is very useful for decontamination of highly polluted with organics wastewaters and municipal wastes.. However, using a variety of wastes and residuals as substrates and mixed cultures in the bioreactor makes AD one of the most complicated biochemical processes, employing hydrolytic, acidogenic, hydrogen-producing, and acetate-forming bacteria as well as acetoclastic and hydrogenoclastic methanogens. Hydrogen; volatile fatty acids (VFAs), including acetic, propionic, isobutyric, butyric, isovaleric, valeric, and caproic acid; and other carboxylic acids, such as succinic and lactic acids, are formed as intermediate products. As these acids are important precursors for various industries as mixed or purified chemicals with high market value, the AD process can be bioengineered to produce VFAs alongside hydrogen, and therefore, biogas plants can become biorefineries [1].

Certain organic wastes, however, cannot be easily digested due to their low nutrient level, which is insufficient for anaerobic digestion; thus, co-digestion (AcoD) (digestion of mixtures of organic wastes) is a viable option. Numerous studies have shown that using co-substrates in AD systems improves methane yields as positive synergisms are established in the digestion medium, and the supply of missing nutrients are introduced by the co-substrates. Synergistic effects among different chemical components under the AcoD process played an important role in improving its performance [2–4]. Biomethane is produced via an interaction between archaeal and bacterial communities.

In our laboratory experimental studies involving co-digestion of mixtures of milk whey and waste activated sludge from the Sofia wastewaters treatment plant (WWTP) in a two-stage process, we obtained a degree of biodegradation (DBD) of 95% [5].

AD is a multi-step biotechnological process with H_2 as a non-accumulating intermediate product [6]. Recently, the interest in H_2 production through AD has increased [7–9]. This is due to the fact that the rates of H_2 production are rather high, and a variety of feedstock can be used as a substrate. In traditional AD, H_2 is consumed immediately, e.g., by hydrogenotrophic methanogens to produce CH_4 and CO_2 [10]. On the other hand, H_2 can be produced separately by engineering the process conditions. However, the main limitation of dark fermentative H_2 production is the rather low energy recovery. In order to completely utilize the organic acids produced during dark fermentation and improve the overall energy conversion efficiency, a two-stage AD concept (TSAD) consisting of a hydrogenic process followed by a methanogenic process has been suggested [9,11,12].

Anaerobic digestion, in particular, two-stage processes, can ensure biological hydrogen and methane productions using organic waste and waste effluents as a feedstock [13]. In addition to economical concerns, the payback time of the TSAD process has been previously determined at around 2–6 years depending on the disposal costs of organic wastes/residues [14].

Some review papers in this field are known. The first review of TSAD [15] combined the optimization approaches for methane, VFAs, and biohydrogen production from the AD. In this review study, the types and configurations of the bioreactors are discussed for each type of product. It is outlined that adapting the same anaerobic systems for VFA, biohydrogen, and methane individually or simultaneously could significantly improve economic and environmental sustainability. The general approach of biohythane production via TSAD is outlined on the base of 132 references [16]. In the same year, it was outlined that at present, TSAD technology is in its testing stage, and according to some researchers, it is not suitable for some kinds of waste processing [17]. A review of anaerobic co-digestion, including the effect of TSAD processes, was presented [18]. A review on TSAD options for optimizing the municipal wastewater sludge treatment process is known [19]. This article provided a guide for the implementation and practical applicability of the TSAD for wastewater treatment plants. A review on microbial biogas enrichment possibilities was presented [20] with a focus on the CO_2 utilization technique, which converts CO_2 into CH_4 through specific hydrogen utilizing microbial species. It was outlined that the TSAD strategy could be the best strategy that could suitably upgrade the existing AD systems to hydrogen-assisted pathways. A review evaluating the scenario and viability of the multi-stage AD process applied to agroindustrial effluents is presented [21]. It is outlined that the major challenges are focused on the stability of the composition and yield of hydrogen in the acid phase, besides the problems resulting from the treatment of complex residues. An overview on biohythane production in Europe at a Technology Readiness Level greater or equal to 5 (technology validated in relevant environment) is provided [22]. This review paper focused on examples of TSAD applications, planned and existing, for treating organic waste of different origins. It discussed how the substrates' composition affects process yields. The utilization of various sugarcane-based industrial wastes by TSAD for sequential biohydrogen and methane production were comprehensively discussed in [23]. Factors influencing TSAD process performance were discussed in detail. The potential of TSAD to reduce emissions of greenhouse gases was demonstrated. Recent findings, implications, and promising future research related to TSAD, including the integration of meta-omics approaches, gene manipulation and bioaugmentation, and the application of artificial intelligence, were highlighted. A recent review elaborated the mechanisms of the two-stage AD process and evaluated recent research trends on this topic [24].

However, most of these reviews discussed particular cases.

This review aims to present the state of the art, advantages, disadvantages, and some perspectives (based on the available literature and our own studies), including all aspects of TSAD—the influence of different parameters, types of bioreactors, microbiology, mathematical modeling, automatic control, energetical considerations, and areas for future research on TSAD processes.

2. Two-Stage Anaerobic Digestion Description

In the TSAD system (Figure 1), relatively fast growing acidogens and H_2-producing microorganisms are grown in the first stage, the hydrogenic bioreactor (BR_1 with working volume V_1). They are involved in the production of VFAs and H_2. On the other hand, the slow growing acetogens and methanogens are grown in the second stage, the methanogenic bioreactor (BR_2 with working volume V_2), in which the produced VFAs are further converted to CH_4 and CO_2. It is known that the overall energy recovery results are significantly higher for the two-stage H_2 + CH_4 system as compared to the traditional one-stage CH_4 production process [9].

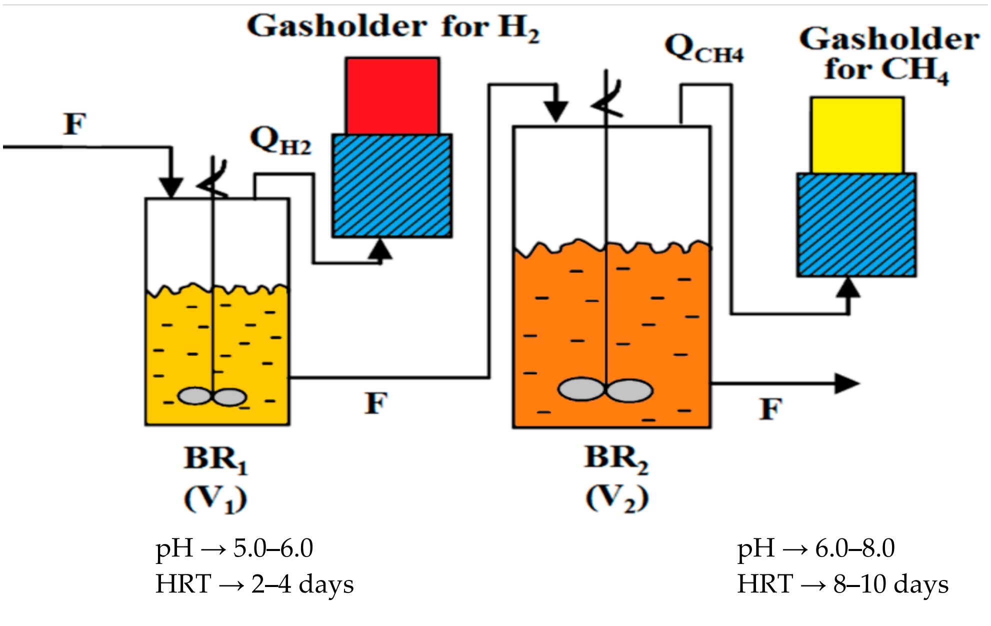

Figure 1. Scheme of the two-phase process of AD with simultaneous production of H_2 and CH_4 [25].

We assume that the volumes V_1 and V_2 of the bioreactors are constant; let F_1 and F_2 be the inflows in the first and in the second bioreactor, respectively; and let $F_1 = F_2 = F$ be valid. It is well known that the dilution rates D_1 and D_2 are defined as:

$$D_1 = F/V_1,\ D_2 = F/V_2,\ \text{then}\ D_2 = (V_1/V_2)\,D_1 = \gamma\,D_1\ \text{within}\ \gamma = V_1/V_2 \quad (1)$$

It is known that the volume V_2 of the second bioreactor for methane production is bigger than the volume V_1 of the first bioreactor. Therefore, the constant $\gamma < 1$ should be valid.

The TSAD process is based on the different activities and relations between acidogens and methanogens in terms of physiology, nutrition needs, growth kinetics, and sensitivity to environmental conditions. In the first stage, the substrate is transformed into H_2, CO_2, VFAs, lactic acid, and alcohols by acidogens with an optimal pH of 5–6 and hydraulic retention time (HRT) of 1–3 days. In the second stage, the remaining VFAs, lactic acid, and alcohols in the bioreactor producing H_2 effluent are converted to CH_4 and CO_2 by methanogens under an optimal pH range of 7–8 and HRT of 10–15 days.

The bottleneck problems occur in the first stage (production of hydrogen) of the TSAD. In this process accomplished by a mixed anaerobic and facultative bacterial population, various metabolic pathways can be simultaneously present during H_2 production.

The main aspects of TSAD have been confirmed with experimental tests, either in batch or in continuous modes, using two feeds representative of organic refuse [26]. The experimental results showed that the energy produced as hydrogen and methane gases was higher than the harvested energy using a one-step AD; specifically, it was in the range of 1.5–2.7 times higher.

The combination of biohydrogen and biomethane production from organic wastes via TSAD could yield a biohythane gas composed of 10–15% H_2, 50–55% CH_4, and 30–40% CO_2 with the high potential to be used as vehicle fuel [9,16].

3. Feedstocks (Substrates)

An enormous quantity of organic waste produced by agriculture, industry, and domestic processes are treated by TSAD processes:

1. Activated sludge (AD and AcoD) [2,4,27–34];

A TSAD of mixed sludge in different volume ratios was investigated. The maximum cumulative H_2 yield (100.5 mL) and CH_4 yield (2643.6 mL) were obtained in a volume ratio of 1:3 (primary sludge: secondary sludge) with an H_2 content of 54.7% and CH_4 content of 59.8%. The organics released in the methanogenic stage were better than in the hydrogen production stage [32].

2. Municipal wastewater [19,35,36] and an organic fraction of municipal solid waste [8,37–42];
3. Industrial wastes [43–47], including molasses wastewater [48];

Hydrogen and methane were simultaneously produced from molasses wastewater by the two-stage system composed of a CSTR and an IC reactor [48]. An HRT of 6 h (D = 0.167 h^{-1}) for hydrogen and an HRT of 12 h (D = 0.083 h^{-1}) for methane production with an OLR of 30 kg COD/(m^3·d) was employed as the optimum condition in the two-stage system. Through the continuous two-stage process for hydrogen and methane production, a maximum of 71.06% of the energy of the molasses wastewater was converted to biogas. In the CSTR-IC two-stage system, more than 80% of the total energy came from methane, and hydrogen had a much lower energy recovery rate than methane. This could have been due to the lower heating value per volume and the relatively lower production rate. Syntrophic propionate-oxidizing bacteria and syntrophic butyrate-oxidizing bacteria in a methane production reactor oxidized propionate acid and butyric acid into acetate and synthesized ATP through substrate-level phosphorylation and methanogens using acetate to produce methane. The pH of the CSTR is stable between 4.31 and 4.62, which was a desirable pH range for both ethanol-type fermentation and hydrogen production. The desirable pH for methane production is approximately 7. The inner pH of the IC reactor was between 6.94 and 7.38, which was a desirable pH for the methanogens. The ORPs in the CSTR and the IC reactor were approximately stable at −430 and −645 mV, respectively. The CSTR-IC system was operated at 35 °C, and the pH was not artificially controlled in the CSTR, while the influent pH of the IC reactor was controlled at 7 by adding NaOH (4 M). After the hydrolysis phase in the CSTR, the IC reactor had a lower OLR, and the average influent OLRs in each stage were 6.093, 7.86, 9.496, and 11.97 kg COD/(m^3·d). The volumetric methane production rates (VMPRs) of each stage were linear, with influent OLRs of the IC reactor, which were 1.32, 1.74, 2.17, and 2.4 L/(L·d). During the fermentation progress, the methane content was between 57.32% and 74.51%, and carbon dioxide and sulfur dioxide could also be detected in the fermentation gas. The CSTR was stabilized as the first stage of the two-stage anaerobic process, and no methane was detected throughout the experiment, mainly because the pH and HRT were both controlled at extremely low values not suitable for methanogens to grow.

4. Agroindustrial waste-milk whey, sugarcane vinasse and leaf, manipueira, vinasse, tequila vinasses, highly concentrated winery effluents, poultry manure, dairy manure, dairy wastewater, and abattoir wastewater [11,21,23,49–59];

To enhance the energy recovery from sugarcane leaf (SCL) through TSAD, the influence of hydraulic retention time (HRT) was investigated [58]. Optimal conditions established through batch experiments (5% total solids (TS) (*w*/*v*) and rice straw compost inoculum) were applied in semi-continuous STRs. The highest production rates were achieved with HRTs of 5 days for CSTR-H_2 (60.1 mL-H_2/L.d) and 25 days for CSTR-CH_4 (238.6 mL-CH_4/L.d). Utilizing SCL for TSAD could reduce greenhouse gas (GHG) emissions by 2.88 Mt-CO_2 eq/year compared to open-field burning. These findings suggest that TSAD has potential in agricultural waste utilization, renewable energy production, and mitigation of air pollution, contributed by the sugarcane preharvesting process.

5. Sugarbeet [60];
6. Slaughterhouse blood waste [61];
7. Cassava [62];
8. Grass and maize silage [63,64];
9. Organic solid waste [38,65–67];
10. Palm and olive mill effluents [45,46,68–70];
11. Paperboard mill wastewater [71];
12. Food waste and food wastewater [12,72–83];
13. Organic market waste [84];
14. Kitchen waste [85] and waste cooking oil [86];
15. Fruit and vegetable waste [74,87,88];
16. Coffee waste [89];
17. Pharmaceutically active compounds [90];
18. Residual fermented solids obtained after biodiesel production [52] offer the possibility of integration among three biofuels of industrial interest (biodiesel, biohydrogen, and biomethane) according to the biorefinery principles, which target the maximum utilization of biomass to produce a variety of products;
19. Mixtures (co-digestion) of organic waste [3,91–99], such as swine manure and rice straw [54]; abattoir wastewater, heterogeneous fruit and vegetable solid waste, and their combination [85]; waste cooking oil and sewage sludge [100]; restaurant food waste and vinasse, a waste from the sugarcane industry [101]; and sewage sludge and waste from the agri-food sector (poultry manure and vinasse).

All authors concluded that the chemical nature of the substrate greatly influences the process and the optimal pH for acidogenesis. Each type of biomass contains a great variety of organic and inorganic elements that considerably affect the digestion process. The increase in biogas production is directly dependent on the content of the substrates submitted to this process [102].

Not only the quality but also the number of solids in the substrates subjected to digestion considerably affect the whole process [103]. Digestion systems for biogas production may be classified according to the load of total solids in liquid digestion, where the content of total solids (TS) is less than 15%. Solid digestion, with levels above 15% [104,105], are classified as wet (TS of the raw material ≤10%), semi-dry (TS of the raw material 10–20%), and dry (TS of the raw material ≥20%) digestion.

In general, liquid digestion systems present higher intensity reactions with short substrate retention times in the reactors, while digesters operating with solid digestion present lower reactor volumes (due to lower dilution of the diluted substrates) but also lower energy requirements [106].

Carbohydrates are the best substrate for this process. Many organic refuses consist not only of carbohydrates but also of complex colloidal particles such as proteins and lipids. Agricultural crops often have high content of water and sugar and are well suited as substrates for fermentation. In recent years, an interesting substrate for the TSAD process has become lignocelluloses in the organic waste [107].

4. Process Parameters and Configurations in Biohythane Production

4.1. Reactor Configurations for Biohythane Production

Different bioreactors have been used as hydrogen or methane production reactors in TSAD. More than 95% of them are operated with CSTRs [29,31,55,58,61,89]. However, many studies are dedicated to new types of bioreactors:

1. Bioflm reactors [108];
2. Membrane bioreactors [109];
3. Upflow anaerobic sludge blanket (UASB) reactors [36,50,64,110] and fixed-bed upflow bioreactors [57];
4. Anaerobic fluidized bed reactors (AFBRs) [46,56];
5. Solid-state anaerobic bioreactors [37] with a first-stage solid-state digester and a second-stage liquid digester;
6. Anaerobic sequence batch reactors (ASBRs) [54].

These studies were performed in laboratory conditions and are too specific, which does not allow their generalization. The results are different and do not allow for industrial implementation. The most interesting results were obtained with a combination of different types of bioreactors in both phases. Some results are shown in Table 1.

Table 1. Some results for existing combinations of bioreactors in TSAD.

BR_1	BR_2	Some Results	References
UASBR (thermophilic conditions)	CSTR (mesophilic conditions)	Optimum conditions for palm oil mill effluent treatment: BR_1—HRT = 22 days; OLR = 275 gCOD/L.day; Q_{H2} = 215 L H_2/kgCOD BR_2—HRT = 5 days; Q_{CH4} = 320 L CH_4/kgCOD; total energy of 15.43 MJ kgCOD; total COD removal efficiency = 94%	[45]
		Optimum conditions: BR_1—HRT = 4.1 h; pH = 5.5 ± 0.1; T_0 = 35 ± 1 °C; COD removal efficiency = 19 (%) for OLR = 29.3 kg COD/(m^3 d); SO_4^{2} removal efficiency = 84 (%); BR_2—HRT = 6 h; pH = 7.2–7.5; T_0 = 35 ± 1 °C; Q_{CH4} = 4.91 L/L.d); CH_4 content = 63% for OLR = 16.1 kg COD/(m^3 d); COD removal efficiency = 95 (%)	[111]

Table 1. *Cont.*

BR_1	BR_2	Some Results	References
CSTR	ASBR	Optimum conditions for palm oil mill effluent treatment: BR_1—HRT = 3 days with Q_{H2} = 106.13 mL H_2/g-COD_{added} for OLR = 0.5 g-COD; BR_2—HRT = 35 days for Q_{CH4} = 334.56 mL CH_4/g-COD_{added}; COD removal = 66.27%; Energy yield (CH_4 + H_2) achieved from TSAD is approximately 38.95% higher than single-stage AD	[69]
CSTR	UASBR	Treatment of Baker's yeast wastewater containing about 20 g COD/L organic compounds: OLR = 1.55 mg COD/cm^3—COD removal = 9.05%; OLR = 4.1 mg COD/cm^3—COD removal = 35.98%; Q_{biogas} = 113.4 L for 40 days	[44]
		For food waste, COD removal efficiency = 96%; for OLR = 15.8 g COD/L.d; Q_{CH4} increased to 5.5 L/L day	[81]
		Optimal ratio D_1/D_2 = 5333 for hydrolysate of agave bagasse	[51]
Solid-bed reactor (thermo- and mesophilic conditions)	UASBR (thermo and mesophilic conditions)	To treat solid potato waste completely within a short period of time, thermophilic conditions are preferred (OLR = 36 g COD L/L.day), but to obtain higher methane yields (Q_{CH4} = 0.49 L CH_4/gCOD/L degraded), mesophilic conditions are preferable	[88]
CSTR	IC reactor	Optimum conditions: BR_1—HRT = 6 h for Q_{H2} = 2.41 L/L.day with a H_2 content = 42% for OLR = 30 kg COD/m^3.day; BR_2—HRT = 12 h for Q_{CH4} = 2.4 L/(L.day) with a CH_4 content = 74.45% for OLR = 36 kg COD/m^3 day	[48]

Nomenclature: CSTR—continuous stirred tank reactor; ASBR—anaerobic sequencing batch reactor; IC—internal circulation reactor; HRT—hydraulic retention time; OLR—organic loading rate; Q_{H2}—maximum volumetric hydrogen production rate; Q_{CH4}—maximum volumetric methane production rate; COD—chemical oxygen demand.

4.2. 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) to thermophilic (55–60 °C) to extreme thermophilic (70–80°C) conditions. Most of the H_2 dark fermentation studies have been conducted at temperature ranges of 35–45 °C.

Temperature is also a very important operation factor in the second stage of the anaerobic digestion process. It determines the rate of the AD 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 conditions [33]. As the temperature increases, the process is much faster, and this results in more efficient operation and lowers the retention time requirements [112]. Thermophilic conditions also lead to an increase in the thermodynamic favorability of CH_4-producing reactions, decrease solubility of CH_4 and CO_2, and cause destruction of pathogens in the reactor effluent. Methanogens are extremely sensitive to changes in temperature, and even a small temperature variation (2–3 °C) can lead to VFA accumulation.

This decreases the CH_4 production rate for methanogens, especially under thermophilic conditions. Maintaining a stable temperature is important for biohythane production.

High temperatures lower gas solubility and lead to partial hydrogen pressure inhibition. TSAD with thermophilic biohydrogen production in the first stage and mesophilic methane production in the second stage of vinasse treatment is suggested [56]. The two-stage system (hydrogen-thermo + methane-meso) yielded 5.5 kJ/g COD, 41% more than the thermophilic system. This saves costs as well as energy. Thermophilic circumstances can promote methane production in the second stage by improving kinetic conditions, but they can also harm some methanogens. This may lower microbe numbers and interactions. Mesophilic methane production resists organic overload and VFA accumulation [113]. At 55 °C, single-stage and TSAD methane production decreased by 13% and 7%, respectively. At 70 °C, the principal methane-producing species were absent, causing acetate buildup and system failure. Thus, the second methane generation stage should be mesophilic.

The AD process may be classified according to temperature [38,114] as:

1. Psychrophilic (below 20 °C),
2. Mesophilic (20–45 °C),
3. Thermophilic (55–70 °C).

The most interesting results were obtained with a combination of different temperatures in both phases. Some results are shown in Table 2.

Three sets of temperature conditions (with different types of bioreactors as well) have been investigated—(I) mesophilic + mesophilic, (II) mesophilic + thermophilic, and (III) thermophilic + thermophilic—with different optimal results concerning the yields of methane and DBD [88].

Mesophilic bands are interesting because they make up the average temperature of most tropical countries. In addition, they ensure higher process stability and greater diversity of active anaerobic microorganisms [23,106]. Processes conducted at this temperature range are widely used when high methane levels are required, either from the process as a whole or from the digestion phase [31]. In contrast, temperatures encompassed by the thermophilic range ensure higher rates of organic loading and initial hydrolysis of the substrates, reducing hydraulic retention times and providing higher yields of biogas production [106]. Besides the fact that high temperatures limit the number of active microorganisms, it should be noted that the rapid degradation and production of toxic compounds might affect or inhibit the development of methanogenic microorganisms [106]. A sharp increase in temperature results in an increase in the mortality rate of methanogenic organisms compared to the mesophilic phase temperatures [115]. On the other hand, the same conditions also considerably reduced the concentration of pathogens harmful to the process [116], besides being interesting for systems aimed at obtaining hydrogen as a final product. Based on this, two-stage systems can be observed using thermophilic temperatures (55 °C) during the hydrolysis and acidification phase of the material and later mesophilic conditions (35 °C), ensuring higher stability and diversity of methanogenic archaea in the methane production phase [61]. A similar condition was also reported using temperatures of 50 °C for the acid phase and 38 °C for the methanogenic phase [117]. The authors obtained an increase in SV removal from 42% to 55% and an increase in methane production from 280 to 332 L/kg added SV.

Tables 1 and 2 complement each other.

Table 2. Some results obtained with a combination of different temperatures in both BRs.

Temperature in BR_1	Temperature in BR_2	Some Results	References
Mesophilic	Mesophilic	Substrate: protein-rich synthetic wastewater inoculated with anaerobic sludge. The biogas production began to decrease at the protein concentration of 12 g/L. The total VFA and ammoniacal nitrogen concentration increased with an increase in protein concentration in BR_1, while the protein and COD removal percentage was higher in BR_2.	[110]
Mesophilic in CSTR	Mesophilic in CSTR	Substrate: sugarcane leaf; optimal conditions: BR_1—HRT = 5 days; Q_{H2} = 60.1 mL-H_2/L.day; BR_2—HRT = 25 days; Q_{CH4} =238.6 mL-CH_4/L.day; total energy recovery = 4.5 kJ/g-VS.	[58]
Mesophilic in CSTR with recirculation pump	Mesophilic in CSTR with recirculation	Substrate: pharmaceutically active compounds with sewage sludge. Optimal conditions: BR_1—OLR = 1.5 kg VS/m^3 day; Q_{H2} = 0.337 m^3/kg VS; SRT = 14 days; BR_2—OLR = 0.9 kg VS/m^3 day; Q_{CH4} = 0.433 m^3/kg VS; SRT = 29 days.	[90]
Mesophilic in CSTR	Mesophilic in internal circulation reactor	Substrate: molasses wastewater. Optimal conditions: BR_1—OLR = 30 kg COD/(m^3.day; Q_{H2} = 2.41 L/(L.day) hydrogen content = 42%; BR_2—OLR = 36 kg COD/(m^3.day; Q_{CH4} = 2.4 L/(L·d) with a methane content = 74.45%. The maximum of 71.06% of the substrate energy was converted to biogas (hydrogen and methane) at the OLR = 30 kg COD/m^3·day.	[48]
Mesophilic in CSTR	Mesophilic in CSTR	AcoD of restaurant food waste and vinasse, a waste from the sugarcane industry. The TS and TVS of the effluent generated in the first stage were reduced by 52% and 64%, respectively, constituting an excellent substrate for the production of biogas rich in methane (72.7%) in the second stage.	[100]
Thermophilic	Mesophilic	Substrate: waste-activated sludge with antibiotic resistance genes (ARGs). The removal efficiency of TSAD to total ARGs was higher than that of one-stage AD.	[118]
Thermophilic in CSTR	Mesophilic in CSTR with recirculation	AcoD of swine manure (SM) and rice straw (mixing ratio of 3:1); Q_{CH4} = 0.44 ± 0.03 L/L.day; digestate recirculation increased total CH_4 production, organic matter removal, and reaction by 9.92, 5.22, and 9.73–12.60%, respectively. The energy input of the system increased by 30.26%, and digestate recirculation improved the energy balance of the total system by 6.83%.	[92]
Thermophilic in CSTR	Mesophilic in CSTR	Substrate: mixture of 50% sewage sludge and 50% wine vinasse. Maximum Q_{CH4} = 1.8 L/L·day) at HRT = 2 days, maximum specific Q_{CH4} = 159.4 mL CH_4/g COD removed and archaea activity (11.6·10^{-9} L CH_4/cells) at HRT = 4 days.	[98]
Thermophilic in AFBR	Mesophilic in ATFBR	Substrate: sugarcane stillage. This combination achieved the best energetic yield: 5.5 kJ/g COD, which is 41% higher than in single-stage system for OLR 24.7 kg COD/m^3.day.	[56]
Thermophilic in BCAR	Mesophilic in BCAR	Substrate: poultry manure. Optimal performance obtained for OLR = 7.5 (g COD/L.day), COD = 43.0 (g COD/L), HRT = 5.75 (day).	[49]

Table 2. *Cont.*

Temperature in BR_1	Temperature in BR_2	Some Results	References
Thermophilic in UASBR	Mesophilic in CSTR	Optimum conditions for palm oil mill effluent treatment as in Table 2. The overall energy recovery was higher than one-stage hydrogen production and one-stage methane production.	[45]
Mesophilic	Thermophilic	Substrate: highly concentrated winery effluents. Optimal conditions: BR_1: OLR = 120 kg COD/(m^3.day) (ensured the highest acetic acid concentration); BR_2: Q_{CH4} = 7.1 Nm^3 CH_4/m^3.day; 348 L CH_4/kg COD for OLR = 29.9 kg COD/m^3.day. However, a lower removal of organic matter was observed under that condition.	[59]
Mesophilic in CSTR	Thermophilic in CSTR	Substrate: waste-activated sludge. HRT of the BR_1 is a crucial parameter to improve the performance of the BR_2.	[29]
Mesophilic in CSTR	Thermophilic in CSTR	Substrate: waste-activated sludge with low-energy sonication pretreatment. BR1—HRT = 3–5 days; BR2—HRT = 10 days; VS removal = 44 to 55%, (35–40% in single-stage); Q_{CH4} = 0.2 Nm_3/kgVS (+11%).	[119]
Thermophilic AFBR	Thermophilic AFBR Mesophilic AFBR	Substrate: sugarcane stillage. Methane production up to 237% at 24.7 kg COD/m^3day). Methane yield up to 118% at 24.7 kg COD/m^3/day) for both mesophilic and thermophilic second-stage reactors when compared to single-stage methanogenesis.	[56]
Thermophilic dark fermentation reactor	Thermophilic with recirculation	Substrate: waste-activated sludge. Biofuel and bioenergy were best recovered at a recirculation ratio of 0.11; 1.48 L H_2/L.day, 0.88 L CH_4/L.day, 106.2 mL H_2/g VS, 161.3 mL CH_4/g VS, 7.7 kJ/g VS, and 88.2 kJ/L.day were obtained depending on HRT. It has been shown that a low recirculation ratio can improve the performance.	[120]
Thermophilic dark fermentation reactor	Thermophilic elec-tromethanogenic reactor (2.5 V)	Substrate: waste-activated sludge. BR_1—Q_{H2} = 0.11 NL/g VS; H_2 = 52%; BR_2—methane yield = 0.39 NL/g VS (+40.5%); volumetric methane production rate = 1.16 NL/.day (+38.8%).	[121]
Thermophilic AMBR	Thermophilic AMBR (V_2/V_1 = 2)	Substrate: tapioca starch-based synthetic high-strength wastewater. BR_1—OLR = 6–8 kg COD/m^3.day; HRT = 19.45 h; BR_2—Q_{CH4} = 1.5–1.9 L_{CH4}/L.day; HRT = 38.92 h; COD removal efficiency = 89–92%.	[109]
Psychrophilic	Psychrophilic	Higher amount of biogas is produced (0.800 m^3/kgVS) than in mesophilic single-stage AD of food waste.	[80]
HUSBR	UASBR	HRT = 5.7 to 2.8 h for BR_1 and from 13.9 to 6.5 h for BR_2. Good performance obtained for influent COD higher than 250 mg/L, while extreme wastewater dilution by rainfall caused an efficiency cut down.	[36]

Nomenclature: CSTR—continuous stirred tank reactor; UASBR—upflow anaero-bic sludge blanket reactor; BCAR—bubble column anaerobic reactor; AFBR—anaerobic fluidized bed reactor; AMBR—anaerobic membrane bioreactor; SRT—sludge retention time; TS—total solids; TVS—total volatile solids; HRT—hydraulic retention time; OLR—organic loading rate; Q_{H2}—maximum volumetric hydrogen production rate; Q_{CH4}—maximum volumetric methane production rate; COD—chemical oxygen demand.

4.3. pH and Alkalinity

Among all chemical factors influencing dark fermentation, pH is considered the most influential. It affects 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 literature reports, a pH of 5.5 has been considered optimal for H_2 production [70,122,123]. 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. The optimal pH is highly dependent on the microorganism. Controlling of pH and alkalinity of a substrate is essential for the first stage of dark fermentation since organic acids produced tend to decrease the pH. A pH lower than 4.5 tends to inhibit the activity of hydrogenases. A low pH also causes a shift in 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, a low pH can turn the metabolism toward ethanol production [94]. CH_4 production is favored at an alkaline pH, exhibiting maximum activity at a pH of 7.8–8.2 [124]. 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 VFAs, may cause a drop in pH if fed with a high OLR. A pH adjustment can be achieved by the addition of an alkali chemical, typically calcium carbonate or sodium hydroxide. A cheap material like ash can be used to adjust the pH in an anaerobic reactor [125].

The VFAs/alkalinity ratio is a crucial digester status indicator for TSAD systems. The environment and substrate composition affect this ratio. Thus, sudden pH drops disrupt methanogenesis. The substrate or sodium bicarbonate can cause alkalinity. Alkalinity can be characterized in numerous ways, but some reports suggest using intermediate alkalinity to partial alkalinity (IA/PA) as an indication of AD due to its high sensitivity and ease of analysis [126]. The VFAs/alkalinity ratio is a precursor to AD stability and process performance [127]. IA/PA should be 0.3 and VFAs/alkalinity should be 0.4–0.6. Ratios above these ranges imply organic overloading [128]. Although determining alkalinity is simple, it is important to consider the cost of balancing alkalinity.

Recirculation systems reduce alkali addition and make scaling up the process easier. In TSAD, the effluent from the second step of methane production was recirculated to the first stage of biohydrogen production to provide buffer capacity and maintain the pH. Process recirculation increases ammonia, which is poisonous, and lowers hydrogen and methane outputs. Thus, recirculation rate control is critical [129].

4.4. Inocula

The choice of inoculum is a fundamental step for the good performance of the AD process. The use of sludge from digesters or treatment ponds for the degradation of residues of similar characteristics to the substrates of interest makes the systems more efficient and more adapted [130] and may considerably reduce the lag phase time [107], especially in more complex systems. A review seeking to quantitatively correlate the production of methane with the abundance of microbiota in anaerobic digestion processes is known [131].

Developing an enriched inoculum is very important for obtaining H_2 in the first stage of fermentation [23]. In the enrichment process, a selection procedure was applied to selectively promote H_2-producing bacteria and eliminate H_2 consumers.

The introduction of active inoculum leads to successful organic matter degradation processes. Inocula are prepared according to the substrate and conditions used. They are responsible for starting the substrate degradation and are the main factors that influence the two-stage AD process besides substrate and operation parameters, such as pH, temperature, etc. [132].

The inoculum consortia shape the microbial composition of reactors, regardless if the configuration of the reactors is one or two stage [133].

Current studies show that the choice and preparation of the inoculum increase the yield of biogas, such as biohydrogen or biomethane, in anaerobic biodegradation processes [24].

4.5. Hydrogen Partial Pressure

The H_2 partial pressure in the liquid phase is the major factor influencing H_2 production, as a high H_2 partial pressure causes deactivation of the hydrogenase enzyme.

4.6. 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 a low HRT is dependent on the volume of the reactor and the flow rate of the feed. It is generally well known that the H_2-producing bacteria are fast growing. By applying this principle, H_2 was produced free of CH_4 in continuous CSTR feeding with household solid waste at an acidic pH range of 5.0–5.5 and a short HRT of 3 days (D = 0.33 day^{-1}) without any pretreatment to inhibit methanogens contained in the initial digested manure [134].

HRT is the main optimization parameter of continuous H_2 dark fermentation bioprocesses. In CSTRs, short HRTs or high dilution (D) rates can be used to eliminate methanogens, which have a significant low growth rate [123,135]. However, HRT needs to be maintained at a proper level that still gives a D value less than the specific growth rate of H_2-producing bacteria. Generally, a short HRT is considered to favor the H_2 fermentation metabolism [122]. On the other hand, too high of loading rates may result in substrate inhibition effects, improper food to microorganism (F/M) ratios of H_2 producers, or washout of microorganisms [136].

In the 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. HRTs for anaerobic digestion processes are typically in the range of 15–30 days at mesophilic conditions and 10–20 days at thermophilic conditions [137].

4.7. C/N Ratio

The carbon found in degradable organic structures is extremely important for the digestion process, being used directly in the generation of methane molecules, while the nitrogen (mainly from proteins) is a fundamental element in the formation of the bacterial cells involved in the whole process. Therefore, a balance between carbon and nitrogen concentrations is fundamental for the AD process. Ideal C/N ratios are between 20 and 35 [66,138]. Higher C/N ratios may limit the inoculum renewal and new cell formation, while very low ratios (high amount of nitrogen as ammonium concentrations) may increase environmental toxicity to the microorganisms of interest [41,93]. Both conditions are detrimental to biogas production. Mixing substrates of different properties may help equalize these essential elements. In addition, good pH stability might ensure a reduction in ammonia toxicity in the reactors [139].

For paperboard mill wastewater AD in a multi-phase anaerobic BR, it was demonstrated that reducing the C/N ratio from 47.9 to 14.3 resulted in a decrease in hydrogen yield by 78.65% and an increase in methane yield by 51.56% [71].

Microbial activity is affected by nutrients and substrates. The TSAD of sugar and ethanol industrial wastes is hindered by their imbalanced carbon-to-nitrogen (C/N) ratio, which results in a relatively low overall energy recovery. For efficient usage, these wastes' high C/N ratio (over 40) must be adjusted to 20–30. Low C/N ratios have high ammonium levels. To maximize the C/N ratio, some researchers have suggested co-digesting these wastes with proteinaceous materials, such as water hyacinth, cow manure, and slaughterhouse wastes. Co-digestion improves system stability and methane yield by encouraging a varied microbial population, improving nutrient balance, diluting hazardous chemicals, and increasing buffering capacity [140].

4.8. Trace Elements

Microorganisms need vitamins and micronutrients such Ca, Mg, Co, Ni, Cr, Zn, and Fe.

Biohydrogen and biomethane production require 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 processes. Metals are an essential supplement in the media for dark fermentation. These micronutrients might be required in trace amounts, but they have an influential role as cofactors, transport process facilitators, and structural skeletons of many enzymes (Fe-Fe hydrogenase and Ni-Fe hydrogenase) involved in the biochemistry of H_2 formation [141]. Therefore, several researchers have studied the effect of supplementation of the Fe ion on biohydrogen production.

A detailed analysis of the effectiveness of trace elements (such as Mg, Na, Zn, K, I, Mn, NH_4, Ni, Ca, Cu, and Mo) for microorganism life processes has been performed [60]. Magnesium is the most important nutrient, followed by sodium, zinc, and iron. The presence of iron in certain concentrations is an essential element for the reproduction and activity of bacteria, considering most of the enzymes involved. Parameters affecting HPB activity in hydrogen production contain iron–sulfur clusters, as previously described; an increase in sulfate to 3000 mg $SO_4^{2-} L^{-1}$ inhibits the production of hydrogen due to a change in the metabolic pathway towards butyrate and ethanol.

4.9. Recirculation of the Effluent of a Methanogenic Reactor

Recirculation of the effluent of a methanogenic reactor (digestate) is often used as an option instead of dosing alkali to raise the pH in the hydrogen-producing bioreactor and control it at a fairly high level of ~5.5, which is considered optimal for microorganisms that produce hydrogen and has an inhibitory effect on hydrogen scavengers, mainly on methanogenic archaea [120].

Our own study concerned the TSAD of corn steep liquor with simultaneous hydrogen and methane production in a pilot-scale biogas plant with an automatic control system [142]. CSL, a waste product from processing corn grain to extract starch, was used as a substrate in the process of AD with hydrogen production. The corn extract was provided by "ADM Razgrad EAD". All experiments were conducted in two connected bioreactors. The first one had a work volume of 10 dm^3 (for hydrogen production), and the second one had a work volume of 80 dm^3 (for methane production), with the ratio $V_2/V_1 = 10$. Initial experiments as well as inoculum maintenance were carried out in two laboratory-scale bioreactors, one with a work volume of 1 dm^3 and the other with a work volume of 2 dm^3. In all experiments, a mesophilic temperature of 35 °C and continuous stirring mode were maintained. As an inoculum, the liquid fraction from the anaerobic digestor operating at mesophilic conditions was used.

5. Microbiology—Participating Microorganisms in TSAD

Agricultural wastes are used for the manufacturing of biofuels through anaerobic digestion [143]. If these residues are released into the environment without applying a proper disposal procedure, environmental pollution and harmful effects to human and animal health could be caused. Microorganisms with the ability to reuse raw materials by fermentation processes contribute to the recycling of waste, making the environment eco-friendly [144]. Bacteria are now being explored in the enzymatic hydrolysis of lignocellulosic materials due to their high rate of enzyme production, expression of multi-enzyme complexes, tolerance to extreme environments, and ability to be genetically engineered [145]. Microbes are responsible for the efficient breakdown of complex organic molecules by a series of biochemical transformations [82]. A number of microorganisms are reported to have the capability of degrading and utilizing cellulose and hemicellulose as carbon and energy sources. Realization of a TSAD is a significant breakthrough in the creation of renewable and sustainable energy technology that possesses the potential for transformation of different complex organic wastes into biohythane and simultaneously coping with the threat of energy crises while addressing waste disposal.

The two-stage anaerobic system provides optimal process stability, leads to increased energy efficacy, and permits better control over crucial parameters to ensure effective performance with energy recovery. The microbiome residing in anaerobic digesters drives the AD process to convert various feedstocks to biogas as a renewable source of energy. In a two-stage system, consecutive cooperation of the population of microorganisms enables product synthesis, and they can be further used by another group of bacteria in the next step. The microbiome has been investigated in numerous studies in the last century [54,90,111,118,146]. The diversity of participating microbial communities provides new information on digesters' performance for various biomass valorization and simultaneously biofuel production. With identification of the mixed cultures in reactors for both biohydrogen generation and biomethane production, effective processes could be realized [123].

Most of the feedstocks used for biogas production, such as livestock manure, crop residues (mainly lignocellulosic), and municipal sludge, are complex and rather recalcitrant to microbial hydrolysis, which is the rate-limiting step inherent to these feedstocks. To achieve efficient AD, a diverse microbiome is required [147]. The AD of renewable biomass is a microbial process wherein a community of fermentative and acetogenic bacteria together with acetoclastic and hydrogenotrophic methanogens convert organic matter into CO_2 and CH_4 [148].

The investigation of microbial communities basically includes the study of the community as a whole, operating under as close to the in situ conditions as possible, and the study of the constituent functional groups of microorganisms as interacting components [149]. When involving cellulose-containing substrates, the species that degrade cellulose belong mainly to the genera *Cytophaga*, *Cellulomonas*, *Cellvibrio*, *Bacillus*, *Clostridium*, and *Sporocytophaga* [150]. The strictly anaerobic thermophilic bacterium *Ruminiclostridium thermocellum* is the microorganism with the fastest documented growth rate on recalcitrant substrate crystalline cellulose [151]. These organisms have a remarkable ability to form very large extracellular multi-enzyme complexes known as cellulosomes. Similar complexes are formed by related Clostridia (such as *Clostridium acetobutylicum*, *Clostridium cellulolyticum*, *Clostridium cellulovorans*, *Clostridium josui*, and *Clostridium papyrosolvens*) and other anaerobic cellulose-degrading bacteria, such as *Acetivibrio cellulolyticus*, *Pseudobacteroides cellulosolvens*, and *Ruminococcus albus* [152].

Molecular biology techniques have overcome the limitations of cultivation-based methods and allowed for the identification of unculturable microorganisms, revealing the high diversity of microorganisms involved in AD. Several methods have been applied to investigate their microbial diversity, such as methagenomics in recent years—an efficient method for determining the complex microbiota structure. It has enabled a significant increase in discoveries related to the microbial world—new microbe-based applications in ecology, agriculture, and human health [153].

The data included in metagenomic analyses generally originate from DNA sequences in different environments and are generated using next-generation sequencing methods such as Illumina, PacBio, and Oxford Nanopore Technologies (ONT) [154].

In recent studies of AD processes, degradation was proven to be most likely due to the presence of members of the genera *Clostridium*, *Bacteroides*, and *Ruminiclostridium*. Among them, the most widespread species are *Clostridium butyricum*, *Bacteroides oleiciplenus*, and *Ruminiclostridium papyrosolvens* [155]. According to [156], *Bacteroidetes*, *Firmicutes*, and *Protebacteria* were the most abundant phyla, accounting for over 60% of the total relative abundance in two-phase systems and over 40% in single-phase, while all detected *Archaea* belonged to the class *Methanomicrobia* of the phylum *Euryarchaeota*, which are known as methanogenic microorganisms. In many studies, the microbial community analysis has provided crucial information to understand the anaerobic digestion process, which may help improve its efficiency [157–160].

The TSAD process is based on the differences between acidogens and methanogens in physiology, nutrition needs, growth kinetics, and sensitivity to environmental conditions. Acidogens and methanogens are enriched separately in two tanks, enabling optimized growth by maintaining proper environmental conditions in each reactor [29,161].

Hydrogen-producing bacteria differ from methanogenic archaea in terms of the following:

(1) Physiological characteristics: Hydrogen producers can form spores under stress, many pretreatment methods such as heat, chemical addition, and pH shock, and they have been used for the screening of hydrogen producers.
(2) Growth conditions: Hydrogen producers require slightly acidic conditions, while methanogens prefer a neutral pH.
(3) Growth rate: Hydrogen-producing bacteria typically have a faster growth rate compared to methanogens.

While most anaerobic reactors do not provide ideal conditions for acidogenic and methanogenic microorganisms [162], when the stages are separated, the different microbes receive their suitable conditions. When two-step processes are used, the acidification and methanogenesis phases are physically separated [82]. Methanogens possess a thinner cellular membrane and are more susceptible to inhibitors than fermentative bacteria and thus require more stable operation conditions [73]. Growth and activity of different microbes can be enhanced at their optimum conditions in the two stages and thus show better performance [24]. The results from [133] showed the differentiation of the microbially dominant families in the two-stage setup, with *Defluviitaleaceae* and *Clostridiaceae* in the acidogenic and methanogenic reactors of the system, whereas *Dysgonomonadaceae* was used in the single-stage setup.

Microorganisms involved in the first stage (H_2 production) and in the second stage (CH_4 production) of a two-stage anaerobic digestion process are shown in Table 3.

Table 3. Microorganisms involved in the first stage (H_2 production) and the second stage (CH_4 production) of a two-stage anaerobic fermentation process [16].

Stages	Mesophilic Conditions (30–35 °C)	Thermophilic Conditions (55–60 °C)	Extreme Thermophilic Conditions (70–90 °C)
1st: hydrogen production (Bacteria)	*Clostridium* sp. *Enterobacter* sp. *Citrobacter* sp. *Bacillus* sp.	*Thermoanaerobacterium* sp. *Clostridium* sp. *Thermoanaerobacter* sp.	*Caldanaerobacter* sp. *Caloramator* sp. *Thermotoga* sp.

Table 3. *Cont.*

Stages	Mesophilic Conditions (30–35 °C)	Thermophilic Conditions (55–60 °C)	Extreme Thermophilic Conditions (70–90 °C)
2nd: methane production (Bacteria)	*Clostridium* sp. *Bacillus* sp. *Desulfobacterium* sp.	*Clostridium* sp. *Thermoanaerobacterium* sp. *Desulfomicrobium* sp.	*Caloramator* sp.
2nd: methane production (Archaea)	*Methanobacterium* sp. *Methanoculleus* sp. *Methanospirillum* sp. *Methanococcus* sp. *Methanobacter* sp.	*Methanothermobacter* sp. *Methanosarcina* sp.	*Methanothermus* sp. *Methanothermococcus* sp.

The digester design and process need to reach the most favorable conditions, taking into account the differences between the vital conditions of hydrogen-consuming bacteria (HCB) and hydrogen-producing bacteria (HPB). The best operating conditions permit the inhibition of HCB and thus allow HPB to become the dominant population. The currently known differences between HCB and HPB concern their resistance to temperature, extreme environmental conditions, and the different growth rates of each species [9]. Bacteria belonging to the genus Clostridium are the main ones responsible for H_2 production. They are obligate anaerobes and are Gram-positive and rod-shaped. Clostridium spp. have a substantial characteristic that distinguishes them from other bacteria, allowing for the production of bioH_2 in anaerobic processes instead of bioCH_4: they are capable of producing protective end spores by undergoing a process called sporulation. This occurs when bacteria are exposed to harsh environmental conditions for bacterial growth. To be precise, endospores are metabolically inactive dormant bodies, like seeds, which wait until the environment again becomes favorable to life. Once environmental conditions change, the endospores germinate back into living vegetative cells that can grow and thrive. In extremely restrictive conditions, the spores might be very resistant and not easily destroyed, as opposed to HCB that are methanogens without such an ability to resist [163]. In a specific case, when Clostridium spores are placed in favorable conditions with nutrients and anaerobic conditions, the germination and metabolism processes can restart [164], and consequently hydrogen and other metabolic products can be produced. *Enterobacter* spp. are also H_2-producing microorganisms with the advantage that they are facultative bacteria able to grow in the presence of oxygen. Based on a phylogenetic analysis of the rDNA sequences in [165], it was found that 64.6% of all the clones were affiliated with three *Clostridium* species, 18.8% with *Enterobacteriaceae*, and 3.1% with *Streptococcus bovis* (*Streptococcaceae*). The remaining 13.5% belonged to eight operational taxonomic units whose affiliations were not identified. Methanogens play a vital ecological role in anaerobic environments by removing excess hydrogen and fermentation products yielded by acetogenic bacteria, producing methane. Methanogens are usually coccoid rods or rod-shaped bacteria. There are over 50 described species of methanogens, which do not form a monophyletic group, although all of them belong to the archaea. Methanogens are strict anaerobes, and when they are exposed to an aerobic environment, the oxygen lowers their adenylate charge and causes their death [166]. The physiological differences between HPB (also called acidogenic bacteria) and HCB (methanogens, archaea, and homoacetogenic bacteria) are the basis of the scientific rationale behind the development of the various methods proposed to prepare hydrogen-producing seeds [34]. The following list summarizes the main differences between HPB and HCB:

- Most methanogens are limited to a relatively narrow pH range (about 7–8) [166], while most HPB can grow over a broader pH range (4.5–7) [34].

- HPB have much faster growth kinetics than HCB.
- HPB are able to resist harsh environmental conditions due to protective spore formation, while HCB are very sensitive and do not have this capacity.

Various methods and techniques have been applied to investigate microbial diversity: the clone library of 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE) analysis, and fluorescence in situ hybridization (FISH) [167]. Metagenomics is an efficient method for determining the complex microbiota structure and performing metabolic mechanism analysis. It is applied for the elucidation of community structure and metabolic pathways analysis to determine the mechanism of cellulose degradation in natural consortia with their synergetic operation. Microbial community engineering is a topic of growing interest within the biotechnological field, which encompasses species–species and species–environment interactions, including mainly symbiotic associations [84]. In the TSAD with wheat straw as a substrate, the first bioreactor contained mainly the genera *Proteiniphilum*, *Prevotella*, and *Clostridium* followed by *Caproiciproducens*, *Dechlorosoma*, and *Caloramator*. The microbial community of second bioreactor was dominated by the genera *Proteiniphilum*, *Bacteroides*, *Anaerotaenia*, *Ruminiclostridium*, and *Hungateiclostridium*. They have the potential to produce methane by acetoclastic or hydrogenotrophic metabolic pathways. In our own research, *Proteiniphilum saccharofermentans* represented 28.2% of the microbial community in the first hydrogen-producing reactor and 45.4% in the second bioreactor. Archaeal representatives belonging to *Methanobacterium formicicum*, *Methanosarcina spelaei*, *Methanothrix soehngenii*, and *Methanobacterium beijingense* were proven in a methane-generating reactor [168]. Microbiological analysis by 16s rRNA sequencing identified *Bacillus* as predominant in CSTR-H_2, followed by *Lactobacillus* and *Clostridium* [23].

The process of AD is dependent on the physicochemical characteristics of the substrates used, but the role of microorganisms is equally important [169]. Most important is the abundance of specific anaerobic microbial species forming the consortia [140]. The correct ratio of key microbes is essential for ensuring stability and efficiency of anaerobic biodegradation. TSAD allows for the production of hydrogen-rich biogas, with hydrogen being addressed as the fuel of the future [170]. Methanogens are also important in the microbial conversion of carbon into methane, which is also a high-energy fuel.

Various strategies for biohydrogen production exist as a demonstration of the taxonomically different metabolic repertoires of the biohydrogen-producing microorganisms that have been isolated [171]. Methanogens produce methane gas and are responsible for more than half of all methane produced on Earth every year; they are part of the limited number of microorganisms that control the flux of biologically generated methane [172].

Future prospects include introducing genetic engineering that will impose its role in all aspects of biotechnology and in biofuel production, together with developing accurate metabolic models to address pathways of interest with a focus on energy supply and waste management, which are two of the great challenges that humanity has to cope with [173].

6. Mathematical Modeling

A lot of models separately describing fermentative hydrogen production [174] and the AD for methane production [175] are known. However, few models of the TSAD process are known from the accessible literature [31,57,176].

A discrete time state space model was used to predict the future behavior of two-phase anaerobic reactor treatment of wastewater and a sludge mixture without the inclusion of hydrogen production [31]. As compared to other models, the proposed model was constantly updated, keeping track of the latest conditions in the reactors. Based on the experiment, the model was able to provide estimations of various parameters in the anaerobic process, including methane production, total and soluble COD, and dissolved solid concentration.

The model was able to estimate increases and decreases in VFA concentrations in both acidogenic and methanogenic reactors, which describes microorganism activity when using organic substrates. A drop in VFA utilization may indicate early signs of process failure. The resulting estimation can then be used for designing a model-based control system to ensure process stability and optimize methane production in anaerobic reactors.

Our team developed mathematical models of the TSAD processes started from simple models [25,177,178]. They are useful for software sensors and the design of automatic control algorithms.

Some of our models were developed on the basis of the well-known mathematical model of IWA ADM1 [179–182]. They are useful for theoretical studies and optimization of the TSAD processes using computer simulations. For example, the so-called "static characteristic" may be obtained analytically or via bay simulations (Figures 2 and 3).

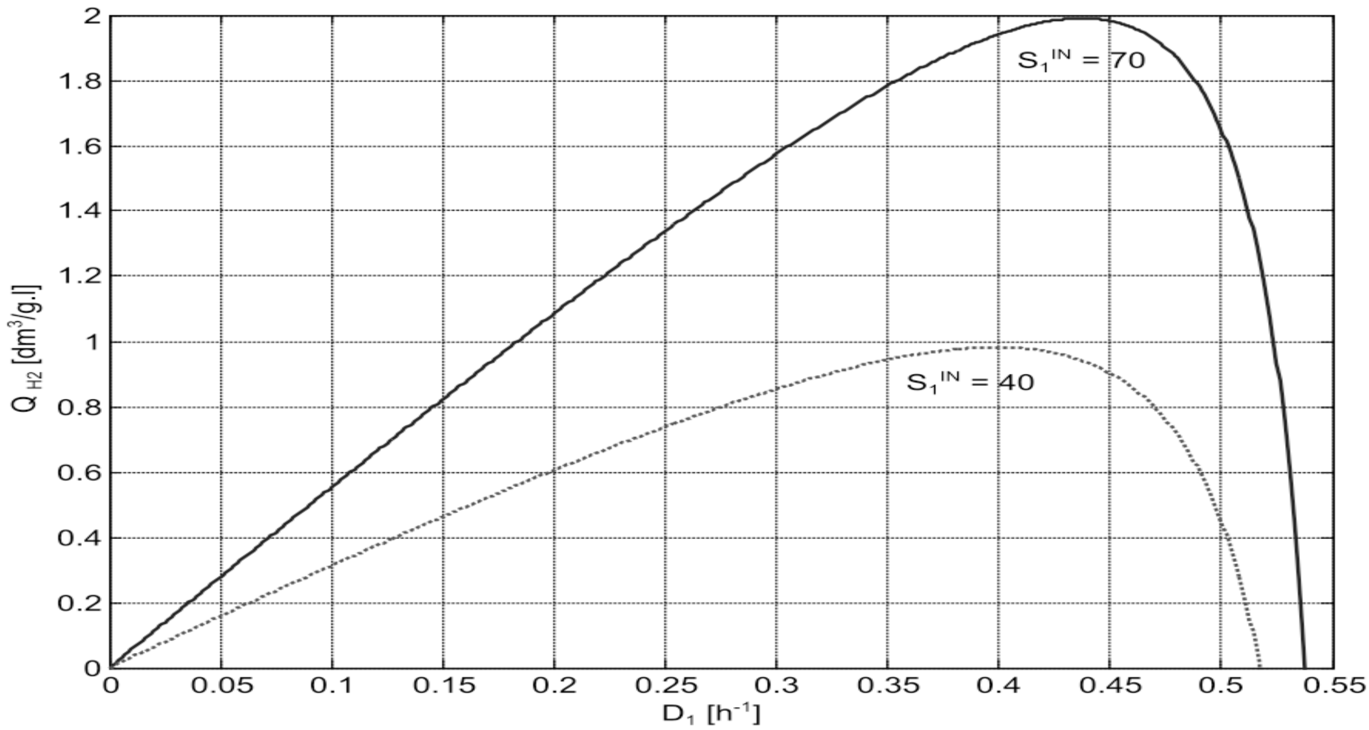

Figure 2. Input–output static characteristic $Q_{H2} = f(D1)$ of BR1.

Recently, some new publications [44,51,183] concerning modeling of TSAD systems have appeared.

The AM2 model was modified with the aim of including soluble COD changes in the effluent near the steady state with the inlet total COD concentration changing at a fixed flow rate at TSAD of an enzymatic agave bagasse hydrolysate without H_2 production [51]. The parameter identification was experimentally implemented online and computationally performed via the Levenberg–Marquardt algorithm. An experimental setup was carried out on a two-stage biomethane production process for instrumented and automated bioreactors.

Recently, new approaches have been compared with traditional mathematical modeling. A popular computational prediction strategy to efficiently model and study non-linear systems is based on self-adapting techniques. One such approach is machine learning (ML), which consists of a group of methods for intelligent data analysis that automate analytical model building. By using algorithms that iteratively learn from data, ML allows computers to find hidden insights without being explicitly programmed on where to look. Computational self-adapting methods (Support Vector Machines—SVM) were compared with an analytical method for effluent composition prediction of a TSAD process [49]. Experimental data for the AD of poultry manure were used. The analytical method considered the protein as the only source of ammonia production in AD after degradation. Total ammonia nitrogen (TAN), total solids (TS), chemical oxygen demand (COD), and total volatile solids (TVS) were measured in the influent and effluent of the process. The TAN concentration in the effluent was predicted, as this is the most inhibiting and polluting compound in AD. Despite the limited data available, the SVM-based model outperformed the analytical method for TAN prediction. Thus, this research showed that:

- When historical data are not available and when no inhibition is detected, the analytical method shows great capability for the prediction of TAN;
- When experimental data are available, ML techniques for modeling complex bio-processes are efficient, as they are capable of predicting and modeling non-linear interactions that are hidden in datasets;
- Since the SVM-based model can be trained with new daily data, the model can improve its performance as new data are gathered, independent of the operational conditions.

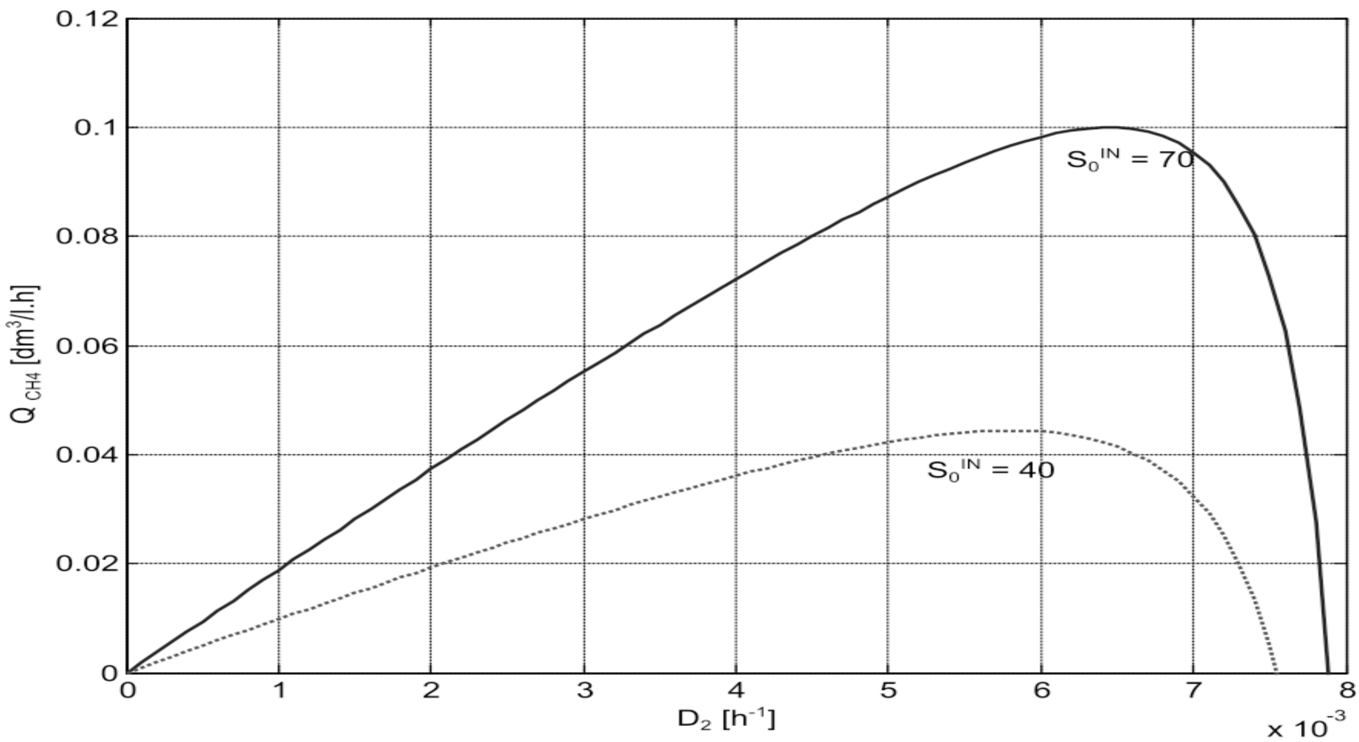

Figure 3. Input–output static characteristic $Q_{CH4} = f(D_2)$ of BR_2.

The development and evaluation of three adaptive network fuzzy inference system (ANFIS) models for a laboratory-scale TSAD systems with UASBR treating high-strength dairy wastewater (yoghurt whey) outputs with varied input selection approaches was presented [184]. The membership functions were obtained from a dataset (85) of the UASBR system, which was first normalized and then divided into 51 data points for training and 34 for testing. The parameter estimation was obtained by applying a hybrid learning algorithm, and the validation of the model was carried out using experimental data of the UASBR system's effluent parameters, such as output pH, COD, and VFA. No pretreatment of the raw data or elimination of the model results was applied. A total of three input variables—pH, COD, and VFAs (from the influent)—and three outputs—pH, COD, and VFAs (effluent)—were adopted. A model was built in the Sugeno structure with the ANFIS editor of the Fuzzy toolbox in MATLAB (R2006 version, The MathWorks Inc., USA). The aim was to investigate the feasibility of the approach-based control system for the prediction of effluent quality from a sequential UASBR system at increasing organic loading rates from 1.1 to 5.5 g COD/L d. ANFIS was successful as modeling unsteady pH data and acceptable for COD within AD limits with multiple input structures. The prediction performance showed a high feasibility for the model-based control system on the anaerobic digester system to produce an effluent amenable for a consecutive aerobic treatment unit. The ANFIS approach used in this study showed that steady conditions at a large OLR range can be modeled with its structure and used to control an anaerobic reactor's influent pH and COD in high-strength dairy wastewaters, where input parameters usually occur at a highly fluctuating level due to dense acidification reactions in the influent. Enlarging the database and/or frequency of monitoring would serve to reduce the error level and improve the predictive capability of the model. On-line and off-line monitoring of the influent pH and COD, respectively, would enable the regulation of the COD concentration in the influent with control and adjustment of a recycled pre-acidification tank using the proposed ANFIS model. As ANFIS can be trained with new data or seasonal changes, the control system based on the model can be adapted or updated continuously by the user, providing great potential for application in the controlling of anaerobic digesters.

A technique to estimate the parameters of a mass balance mathematical model of the TSAD of corn steep liquor for sequential production of H_2 and CH_4 using the metaheuristic crow search algorithm (CSA) was proposed [185]. The process dynamics modeled in the cascade (BR_1 and BR_2) were described by a set of five nonlinear ODEs, representing substrate (corn steep liquor concentration S_1), biomasses (acidogenic bacteria concentration X_1 and methanogenic bacteria concentration X_2), and intermediate products (acetate concentration in BR_1 Ac_1 and in BR_2 Ac_2) adopting Monod kinetics in both of the bioreactors. The flow rates of hydrogen (Q_{H2}) and methane (Q_{CH4}) were represented by two algebraic equations. The model had nine model parameters that were identified. To achieve the best CSA performance, the influence of the main algorithm parameters was investigated. Numerous simulation experiments were performed to find the best tuning of the parameters. Seventy differently tuned CSA algorithms were studied. Boxplots, the parametric test ANOVA, and the nonparametric Wilcoxon test were used to compare the performance of the best developed CSA. The newly proposed InterCritera Analysis was also performed. The best CSA algorithm performance was achieved.

In the known literature, most TSAD processes are operated manually, and the ratio in the working volume of CSTR bioreactors is not discussed. In some reports, a ratio of 10 has been accepted without comment [84]. A ratio of 3.2 has also been accepted without comment [49]. A TSAD system consisting of two CSTRs operating at mesophilic conditions was used to investigate the effect of hydraulic retention time on hydrogen and methane production [186]. The ratio of working volumes of bioreactors was equal to 8 (without comment). Optimization of TSAD with separately collected municipality biowaste in a pilot-scale CSTR with a thermophilic regime using recirculation of the digestate of the second BR to maintain the pH in the first BR at the optimal value was performed [187]. The ratio of working volumes of bioreactors was equal of 3.8. The optimal loading rate was obtained, providing maximum H_2 and CH_4 productions in the TSAD of cassava wastewaters using specific thermophilic bioreactors and a constant recycling ratio of 1:1 with automatic control of pH in the hydrogenic bioreactor [188]. The ratio of working volumes of both bioreactors was equal to 6 (without explanation). In another study, the ratio of working volumes of both bioreactors was equal to 2 (without explanation) [110]. Both biohydrogen and biomethane production were optimized in thermophilic bioreactors using two operating parameters (organic loading rate and dilution rate); however, the first bioreactor was run in semi-continuous and the second in batch operation mode because the working volumes of both bioreactors were equal [189]. The ratio (without explanation) was 2.5 [31].

A review paper [15] combined the optimization approaches for three possible products from AD—methane, hydrogen, and volatile fatty acids (VFAs)—taking into account different process parameters and types of BR, including acidogenesis and methanogenesis separation in two different BRs. However, the ratio of the working volumes of the bioreactors was not included or discussed. Interesting results for the TSAD of cheese whey were presented in [55]. Both bioreactors were CSTR with equal working volumes. The main conclusions from this study were that pH control and a smaller volume of BR_1 should be considered.

In TSAD processes, an important problem exists from a technological and economical point of view—how to determine the optimal ratio of the working volumes of the hydrogen- and methane-producing BRs for optimization of all processes. In [52,87], possible solutions to this problem were presented in view of maximizing the energy production (the H_2 and CH_4 produced). One of the parameters to be optimized for TSAD is the ratio of the working volumes of both bioreactors used. In [142], our team proposed a solution of this problem for CSTR on the base of the developed mathematical models.

7. Control

Recently, the automatic control of TSAD processes has been a subject of increased interest with the application of both classical methods and methods using AI. A theoretical study of inverse optimal neural control via a passivity approach for a TSAD model for simultaneous hydrogen and methane production in the presence of disturbances was presented [190]. The model (consisting of 12 ordinary differential equations) derived on the basis of the ADM1 basic structure is highly nonlinear and sensitive to external disturbances. A recurrent neural network structure to identify complex dynamics of the process and directly related to biofuels (H_2 and CH_4) production was proposed. Inverse optimal control laws via the passivity approach for trajectory tracking based on the neural model were proposed for optimal hydrogen and methane production. The neural control strategy's performance for trajectory tracking in the presence of disturbances was proven. Two cases were presented to verify the optimal control performance: first, reference trajectories based on the input–output characteristic analysis for maximum biofuels production were proposed; second, tracking the performance in the presence of input parameter disturbances was considered. Results via a simulation showed the optimal control methodology efficiency to stabilize the H_2 and CH_4 productions alongside desired trajectories even in the presence of disturbances.

In another study, the control problem was formulated as the TSAD bioprocess operated in an open loop; the acidogenic process yielded VFAs (P_1) at certain concentrations, which increased as the residual substrate decreased, but this requires a very high HRT [51]. In order to solve the above control problem, two Single-Input–Single-Output (SISO) controllers were implemented for each stage. In other words, the feedback control aims to increase the maximum productivity (D_1P_1) with respect to the soluble COD inlet concentration at the acidogenic stage, whereas for the methanogenic stage, the control goal is maximum biomethane production with respect to the VFA inlet concentration. The two controllers were based on the adaptive linearizing controller design [191] with bounded control actions. The proposed feedback controllers were robust in the face of parametric uncertainties with few measurements. They were experimentally implemented and tested. The closed-loop acidogenic stage reached the productivity maxima of the corresponding COD feeding concentrations and improved the process performance compared with the open-loop operation. In a similar fashion, the methanogenic reactor's closed-loop operation improved the biomethane flow production compared with the open-loop operation at different VFA feeding concentrations.

An active fault tolerant control strategy (FTC) for a TSAD process subject to fault actuators was proposed [192]. Due to abnormal operation or equipment aging, actuator or sensor faults occur in these systems. The opportune fault detection and isolation can help with adequate decision making. A recurrent neural network model for unknown nonlinear systems was used to estimate dynamical sates and the fault magnitude. Recurrent neural networks are artificial neural networks (ANNs) that use sequential data, and they take information from prior inputs to generate the next output of the sequence. An inverse optimal control law for trajectory tracking based on the neural model is proposed for fault compensation. Adequate closed-loop control action and reference trajectories were selected to lead the system to an optimal operating order with respect to desirable performance and degree of priority. Two cases were studied: active additive control and degraded performance in the presence of major actuator failures. In the first case, fault-tolerant control was demonstrated to be effective for detecting and compensating actuator faults in two actuators. In the second case, an actuator blocking without redundancy was considered a critical failure. The main objective was to lead the system to safe operation and to minimize the loss of productivity. Results via the simulation showed that the FTC strategy is efficient for stabilizing biofuel production along the desired trajectories in the presence of actuator faults so that the passivation of the entire plant is preserved. A performance guaranteed ultra-local model (ULM)-based predictive control (PG-ULMPC) for TSAD systems was developed [193].

From Figures 2 and 3, it is evident that a maximum biogas yield existed for both BRs (for biohydrogen and for biomethane). This was the basis for the application of the so-called "extremum seeking control". A mini review of our results in this field was presented [194]. The survey paper [195] presented new research results on TSAD systems control obtained by the joint research team of the Department of Biotechnology at The Stephan Angeloff Institute of Microbiology (SAIM) at Bulgarian Academy of Sciences (BAS); the French-Chinese Laboratory on Automatic Control and Signal Processing (LaFCAS) at Nanjing University of Science and Technology; and the Research Center in Computer Science, Signal and Automatic Control of Lille (CRIStAL) at Lille University.

However, most of these algorithms are mainly theoretical achievements, and their practical applicability needs to be proven [196].

8. Energy Considerations

To meet the increased demand for energy needs and to reduce greenhouse gas emissions, the capacity of worldwide installed renewable energy systems has been doubled over the last decade [197]. This also applies to biogas as a source of renewable energy, where the number of biogas plants installed in Europe has increased from 6227 in 2009 to 18202 by the end of 2018 [198]. The total produced electricity from biogas reached 88 TWh in 2017, 40% of which was generated in Germany [198]. Hence, Germany is a leading country in this field. Due to more than 9000 large-scale anaerobic digestion plants, biogas technology is making a significant contribution to the sustainable energy supply in Germany [199]. With a total of around 5901 MW_{el} of installed electrical capacity (on-site electricity generation), electricity generated from biogas amounted to around 31.6 TWh in 2019 and thus accounted for over 58% of total electricity generation from biomass. In Germany, AD plants usually use renewable raw materials and animal excrement (manure and dung) to operate.

Our own experimental results are summarized from an energetical point of view in Table 4 [200].

Table 4. Experimental results obtained during continuous TSAD tests ($\gamma = V_1/V_2 = 0.1$ and $S_{0i} = 50$ g/L) [200].

Hydraulic retention time for H_2 reactor (days)	2.5
Dilution rate D_1 (days^{-1})	0.4
Hydraulic retention time for CH_4 reactor (days)	25
Dilution rate D_2 (days^{-1})	0.04
Hydrogen production (dm^3 L^{-1}day^{-1})	0.241
Methane production (dm^3 L^{-1}day^{-1})	0.405
Total energy production (kWh/day)	0.005028

Our experiments for the TSAD of corn steep liquor are in the initial phase of technology development and have low organic loads on both BRs.

Other representative results [9] for the TSAD of organic wastes are presented in Table 5, where the operative conditions for the continuous tests, the specific hydrogen and methane production (mean value), the total energy harvest by TSAD, and the efficiency values are given.

Table 5. Experimental conditions and results for continuous TSAD tests ($\gamma = 0.1$) [9].

Hydraulic retention time for H_2 reactor (days)	0.25	1	1.5
Dilution rate D_1 (days^{-1})	4	1	0.667
Hydraulic retention time for CH_4 reactor (days)	2.5	10	15
Dilution rate D_2 (days^{-1})	0.4	0.1	0.0667
Hydrogen production (L L^{-1}day^{-1})	1.67	0.49	0.19
Methane production (L L^{-1}day^{-1})	4.52	1.31	0.99
Total energy production (kWh/day)	0.0004997	0.14472	0.108
Efficiency η (%)	49	56	63

The efficiency can be evaluated as η = Produced energy (H_2 + CH_4)/Initial energy embedded in the substrate × 100 (%).

Comparing Tables 4 and 5, it may be concluded that our results for total energy production are close to those presented in [9].

9. Advantages and Disadvantages

9.1. Advantages

In fact, TSAD represents double benefits. The first one is to obtain energy self-sufficiency, and the second is to offer an integral solid and liquid waste management solution with the possibility of river remediation using sludge sediment as the inoculum.

TSAD was proposed because it can degrade most organic pollutants in organic wastewater and solid wastes and obtain renewable energy from hydrogen and methane. Compared with single-stage AD, TSAD has a high organic load rate, high rate of organic removal, and corresponding recycling of heat and electrical energy, which has especially attracted the attention of researchers [17].

TSAD is reportedly superior to single-stage AD in many aspects:

1. Increase in the overall energy recovery by 10–43% [22,201];
2. Reduction of the retention time to 10–18 days;
3. Improvement of the capability of the organic loading rate (OLR) [113];
4. Decrease in the reactor size by 25–45% [129];
5. Diminishing the rate of system failure due to VFA accumulation [202].

Some examples for:

(*A*) *Maximization of the obtained energy*

The methane production rate increased to 5.5 [dm^3/ L day] [81]. In another study [61], a pilot-scale TSAD system treating slaughterhouse blood waste was presented. The conclusions from this were as follows:

(1) A methane yield of 189 mL g^{-1} COD_{added} and a COD removal of 50.8% were obtained at an OLR of 0.4 g COD L^{-1} d^{-1} and operational temperature of 26 °C. The addition of bamboo biocarriers in the digesters increased the methane yield by 103% at this OLR.
(2) *Methanobrevibacter* and *M. beijingense* were the dominant archaea in the system using biocarriers. The authors concluded based on visual observations that significant microbial biofilms were created on these biocarriers.
(3) The authors estimated that 13.4, 24.1, and 19.5 GJ d^{-1} can be recovered from typical slaughterhouses processing bovine, swine, and broiler chickens, respectively.

Treatment of coffee pulp using a TSAD process could increase the methane level production by at least a factor of 3 [89].

(*B*) *Maximiation of the degree of biodegradation (DBD)*

In the CSTR-UASB TSAD process, the COD removal efficiency was consistently over 96% for loading rates up to 15.8 [g COD/L day] [81]. The COD removal efficiency deteriorated at loading rates over 18.7 [g COD/L day] due to sludge flotation and washout in the reactor, which resulted from a short HRT of less than 10.6 [hour].

In the TSAD treatment of raw municipal wastewater at very variable influent conditions (total COD from 401 to 118 [mg/L] and temperature from 21 to 14 °C), the plant was operated at an overall HRT ranging from 9.3 to 17.3 [hour]. Good performance was obtained for an influent COD higher than 250 [mg/L], while extreme wastewater dilution by rainfall caused an efficiency cut down [36].

The methane yield from the TSAD of cheese whey (energetically rich product that can contain more than 50 [g·L^{-1}] lactose) was determined and fluctuated from 136.6 to 216.3 [L_m·$kg_{vs}{}^{-1}$] (L_m—liters of methane, kg_{vs}—kg of volatile solids) [55].

The combination of biohydrogen and biomethane production from organic wastes via TSAD 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 CO_2. The advantage of biohythane over traditional biogas is that it is more environmentally friendly and has a flexible H_2/CH_4 ratio, higher energy recovery, higher degradation efficiency, shorter fermentation time, and high potential to be used as vehicle fuel. Biohythane via TSAD using organic waste could be a promising technology for higher energy recovery and a cleaner transport biofuel than biogas. A H_2/CH_4 ratio in the range of 0.1–0.25 is suggested for biohythane. The flexible and controllable H_2/CH_4 ratio afforded by TSAD is of great importance in making biohythane.

In addition, the TSAD process has the advantages of improving the negative impacts of inhibitive compounds in feedstock, increasing reactor stability with better control of acid production, increasing organic loading rates operation, and significantly reducing the fermentation time [16].

The growing search for alternative and clean sources of energy that present technical and economic viability has driven researchers and enterprises in the biogas production chain to develop new systems, reactors, strains of microorganisms, and optimization of parameters, among other issues. In the treatment of agroindustry effluents, systems using the AD process with physical division of the stages may be a high-value tool for the process, even more so when used synergistically with other factors. High stability, the support for high organic loading rates, the possibility of optimal condition adjustments for each group of microorganisms, and the obtaining of hydrogen fuel are some of the advantages presented by systems that employ multiple stages. Besides the operational advantages and the possibility of using molecular hydrogen, the production of biohythane makes it possible to increase the torque and performance of internal combustion engines, the energy balance, and the environmental gains by reducing smoke emissions. This case has attracted the interest and support of several automotive companies around the world [21].

The thermophilic-mesophilic TSAD configuration was the best in terms of volatile solids removal and methane yield. This configuration was also beneficial for pathogen reduction, digestate dewaterability, and energy and cost effectiveness [19].

(*C*) *Greenhouse gas (GHG) reduction*

The effects of CO_2 capture from combustion flue gas and its use in a TSAD process to improve energy recovery and to reduce CO_2 emissions were reported [30]. In this work, a TSAD process fed with urban wastewater sludge was successfully established and maintained for several months at a pilot scale. The TSAD process with injection of CO_2 exhibited efficient biomass degradation (58% VSS reduction), increased VFA production during the acidogenic phase (leading to a VFA concentration of 8.4 g/L), and high biomethane production (0.350 Sm^3/kg_{SSV}; 0.363 Sm^3/m^3 $_{react}$.d). Moreover, CO_2 intake in the acid phase had a positive impact on the overall GHG balance associated with biomethane production and suggests an improved solution for both emission reduction and biomass conversion into biomethane. Thus, TSAD with a CO_2 injection system can be regarded as a carbon-negative energy technology due to a combination of CO_2 intake and a more efficient biomass degradation.

The carbon footprint represents the total amount of GHGs, expressed in terms of CO_2 equivalent (CO_2-eq), that are directly or indirectly emitted from various processes in manufacturing one unit of a reference product, i.e., ton CO_2-eq/ton sugar. The CO_2-eq is based on the global warming potential of each gas over a 100-year time horizon according to the IPCC (2013). For example, 1 kg N_2O and 1 kg CH_4 correspond to 298 kg and 34 kgCO_2-eq, respectively [203]. However, the specific quantity of GHG emissions from the sugarcane industry can vary widely depending on factors such as sugarcane cultivation practices, the efficiency of the harvesting and milling processes, byproduct management, and regional specifics.

The carbon footprint between a conventional single-stage AD and TSAD of second cheese whey was compared [204]. The study observed that under the specific conditions examined, TSAD exhibited an improvement in electricity and heat generation owing to hydrogen production. This led to a significantly higher potential for GHG emissions reduction, achieving about a 62% reduction compared to a conventional single-stage AD. This reduction was primarily due to the additional production of hydrogen, which improved the performance of an internal combustion engine by 10%. Moreover, the study found approximately 15 times the GHG emissions reduction compared to off-site AD plants operated by third parties located 50 km away. In agreement with these findings, a previous study stated that burning biogas to produce electricity converts all CH_4 into CO_2, which results in a reduction of over 90% in the carbon footprint [205]. Additionally, the potential impact of large-scale AD on global carbon markets is considerable. Under cap-and-trade regulations, these markets encourage the exchange of carbon credits based on GHG emissions at both national and international levels [206]. Large-scale AD could contribute to developing significant tradeable credits by substantially reducing GHG emissions.

(*D*) *Foaming and scum formation*

The effect of protein concentrations on foaming and scum formation using protein-rich synthetic wastewater in a two-stage anaerobic digester was studied [110]. Foams are a collection of persistent bubbles formed when air or gases are introduced beneath the liquid's surface, which expands to surround the gas in a liquid film known as lamellae. It leads to unstable operation and reduced digester working volume, thus decreasing microbial activity and biogas yield. Scum formation leads to physical, biological, and economic failures if left untreated. It often causes pipe blockages (thus requiring frequent cleaning and maintenance), reduces the reactor surface area, and interferes with mixing equipment. Some causes of scum formation are improper mixing, temperature fluctuations, and organic overloading. The foaming tendency, scum production, biogas production, protein, COD removal, total VFA concentration, and ammoniacal nitrogen (AN) concentration were measured to comprehend the findings. The results showed no foaming or scum in the digester; however, sludge residue with high protein concentrations was present. The biogas production began to decrease at a protein concentration of 12 g/L. The total VFAs and AN increased steadily with an increase in protein concentration in BR_1, while the protein and COD removal percentage was higher in BR_2. However, when using a two-stage digester, scum was absent in both BR_1 and BR_2 for all protein concentrations.

9.2. Disadvantages

The productivity of hydrogen is low in the first stage of TSAD. Thus, the corresponding contribution rate of the energy recycled in the form of H_2 is still low compared to the total high removal rate of COD.

Some researchers have shown that TSAD is not suitable for some kinds of waste processing. At the same time, the technology should be chosen based on the C/N ratio of the substrate. At present, two-stage hydrogen/methane digestion technology is in its testing stage. It has higher energy productivity compared with single-phase AD, but its operation cost is higher because of complex technological processes. In this case, the economic benefit of TSAD is doubtful. With further research on superior microorganisms and increased economic benefits, TSAD will be applied widely, and the transfer from lab to application on a large scale will be accelerated [17].

Several problems are still reported regarding the stability of the production and composition of biogas for the acidogenic phase (hydrogen production rates are very variable in most available studies), even in systems with a continuous and constant feeding regime. In addition, the use of two-stage systems using heterogeneous and highly complex residues is regarded as a major challenge [21].

Energy consumption and recovery in AD varies based on the process configuration and operating conditions, such as digester temperature, sludge pretreatment before AD, OLRs, recirculation of effluent, etc. Some AD processes, especially those operated at elevated temperatures, require more energy input than the recovered energy. The ideal AD systems should maximize energy recovery and reduce the use of external energy.

Pumping, mixing, and heating are the primary AD operations that require external energy. In two-phase systems, these costs are expected to be twice as high. Pretreatment and recirculation are the other ones.

A 2 min/hour intermittent mixing has been suggested as an optimal value for efficient and sustainable energy use to produce enough biogas output for the energy system compared to continuously mixed AD and unmixed AD reactors [207].

10. Future Works

10.1. Integrating Meta-Omics Approaches

Comprehensive meta-omics research on TSAD microbial communities is lacking. Functional microorganisms and their functions in TSAD require more meta-omics investigation. The 16S rRNA gene amplicon sequencing is the most popular method for studying TSAD microbial communities [208]. High-throughput sequencing (HTS) technologies like next-generation sequencing (Illumina) and third-generation sequencing (Nanopore and PacBio) have greatly improved sequencing throughput and accuracy, allowing researchers to generate large datasets quickly.

Thus, meta-omics approaches like metagenomics, metatranscriptomics, metaproteomics, and metabolomics can be used alone or in combination to understand the function and dynamics of microbial communities by identifying microbial functionalities and quantifying their proteins and metabolites.

These technologies can infer metabolic pathways and microbial community interactions, filling the knowledge gap between TSAD and large-scale microbial interactions [209]. Multiomic approaches are promising future research avenues, but they require advanced analytical methods, specialized software, and expensive instrumentation, including high-throughput sequencing, mass spectrometry, and enhanced imaging.

Bioinformatics tools are also needed to process and analyze multiomic data [170]. An important factor is the researchers, especially in resource-limited conditions. As technology improves and becomes more widespread, multi-omics approaches may become cheaper, allowing more researchers and institutions to use them.

10.2. Gene Manipulation and Bioaugmentation

TSAD is widely recognized, yet metabolic processes affect gas concentrations and contribution. Understanding TSAD metabolic pathways can help regulate the desirable product production. Metabolic engineering research on biohydrogen production generally overexpresses hydrogen-yielding enzymes like hydA, mcrA, and zwf or suppresses hydrogen-degrading enzymes like hupSL and hypF. Genetic engineering studies have also redirected metabolic electron flux to produce biohydrogen. This is achieved by deleting the genes responsible for the expression of enzymes that produce competitive end products (e.g., frdB, frdD, and adhE). These changes improve metabolic pathway biohydrogen production. PGI gene deletion or HMF gene insertion can increase microorganisms' resistance to toxicity, particularly furfural and 5-HMF.

Over the past decade, major efforts have been made to reduce sensitivity and improve microbial robustness [210]. These findings make gene insertion, deletion, and expression manipulation easy, which can be used to enhance TSAD efficiency. Genetic engineering may improve the microbial community's biohydrogen and methane production and provide a deeper understanding of genes and their expression in TSAD. New genetic engineering methods require further study.

Bioaugmentation is a novel method for enhancing industrial-scale anaerobic digesters. Augmented microorganisms can overcome these constraints or improve upon existing microbial communities.

Proteobacteria have more lignin-degrading enzyme genes. KKU-MC1 bioaugmentation boosted the richness and variety of the microbial community, which raised the methane output of lignocellulose materials such as cassava bagasse, Napier grass, SCB, and FC by up to 42%.

Pre-hydrolysis of the biomass with KKU-MC1 increased soluble substances by 38–56%, improving biodegradation.

Bioaugmentation may boost sequential biohydrogen and methane production in TSAD.

However, the application of bioaugmentation in TSAD, full-scale applications, and online monitoring of bioaugmentation targets are limited. Further studies are required to determine the feasibility of bioaugmentation for commercial applications.

10.3. Artificial Intelligence

ML has grown in popularity for process optimization, performance prediction, and real-time monitoring. This is because conventional theoretical and mathematical models, such as the ADM1, modified Gompertz model, cone model, and first-order kinetic model, are time-consuming and require prior knowledge and parameter calibration [211].

However, ML is good at capturing complicated and nonlinear phenomena that cannot be represented mechanistically [212]. Several artificial intelligence (AI)-based algorithms have been developed and used to optimize process parameters (using a central composite design-RSM (CCD-RSM)), optimize the maximum volume of substrates and travel logistics (using ant colony optimization (ACO), genetic algorithms (GA), and particle swarm optimization (PSO), and optimize and predict the substrate. Several studies have predicted product yield (hydrogen and methane) and gas compositions using AI-based algorithms like the artificial neural network (ANN), tree-based pipeline optimization tool (TPOT), genetic simulated annealing algorithm (GSA), and fuzzy logic models.

TSAD digesters can fail due to environmental factors affecting second-stage methane production. Thus, real-time AD monitoring is essential for maintaining the process and taking immediate action before methanogenesis becomes a crisis. Source [213] predicted AD failure using online sensors and a novel model of combined convolutional neural networks and bidirectional long short-term memory (CNNBdLSTM). In addition, the performances of different models have been compared [214]. Genetic programming (GP) is more accurate than other models for real-time VFA concentration estimation utilizing pH, ammonia concentration, CO_2 fraction, and pressure.

ML's use in AD has been widely studied [211,215]. ML models offer benefits and drawbacks. They need lots of data to avoid overfitting and poor training performance. Second, further research is needed to compare algorithms and find optimal algorithm combinations.

Finally, several ANN models have been criticized for their "black-box features," making their internal mechanics hard to understand [211].

However, ML models in TSAD can reveal complicated parameter relationships and improve the process for efficiency and stability.

Therefore, further studies on the application of TSAD are crucial for future research. Figure 4 shows AD research opportunities.

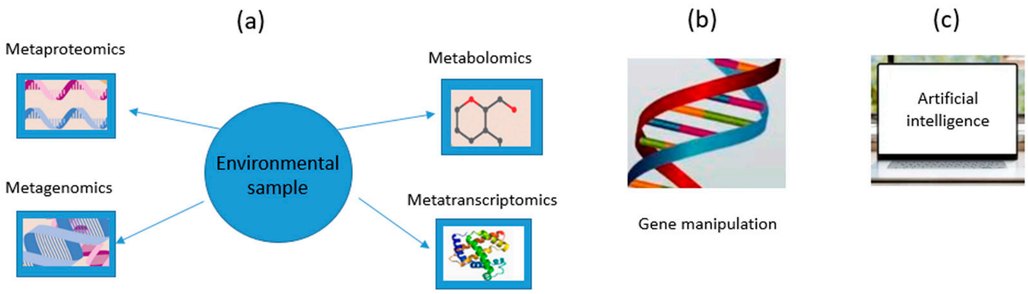

Figure 4. Promising areas for future research in AD include integrating meta-omics approaches (**a**), gene manipulation and bioaugmentation (**b**), and artificial intelligence (**c**) [23].

11. Conclusions

Methane is commonly used not only in chemical industry but also in transport as compressed natural gas, which has been regarded as a clean energy carrier in comparison to gasoline or diesel. However, by combining the advantages of H_2 and CH_4, biohythane is considered one of the most important fuels involved in achieving the transition of technical models from a fossil-fuel-based society to renewable-based society. Biohythane via the TSAD of organic waste could be a promising technology for higher energy recovery and a cleaner transport biofuel than biogas. Various types of organic wastes can be used as substrates for biohythane production. At the moment, there are only a few examples of biohythane plants at pilot or higher scales. The highest volumes of 700 L and 3800 L for acidogenic and methanogenic phases, respectively, were successfully tested close to Milan, Italy. However, with the development and distribution of TSAD systems, a new route for methane and hydrogen generation can be opened.

TSAD could increase DBD, net energy balance, CH_4 production rates, as well as high yield and purity of the products. In addition, the TSAD process has the advantages of improving the negative impacts of inhibitive compounds in feedstock, increasing reactor stability with better control of acid production, improving organic loading rates operation, and significantly reducing process times. However, any AD configuration should be both economically and environmentally feasible before industrial application. TSAD implementation can be simplified through the conversion of current biogas plants with only the addition of another reactor. This would avoid the construction of new plants, a factor that greatly increases implementation costs.

Identifying a correlation between the type of feedstocks to be utilized and the abundance of existing species of the microbial consortia involved, optimizing AcoD systems through the proper selection of feedstocks and their combination ratio could be achieved. In this way, the control of multi-stage systems would be realized, as the different microbial communities, including hydrolyzing bacteria, acidogenic bacteria, and methanogenic archaea, would be selectively enriched in the three separate reactors in each stage.

From a technical point of view, future research targeting the isolation and immobilization of stable and adapted bacteria to the digestion process, together with studies on the combination of specific biodigesters, pre-treatments, and substrate mixtures, are necessary. From an economic point of view, few works have reported installation costs, pay-backs, and feasibility of TSAD-oriented ventures. The development of this sector is fundamental to reach high levels of technological maturity, enabling the scale-up of two-stage digestion, which in most cases occurs only at the bench or pilot scale.

In the opinion of the authors, the most realistic industrial implementation of TSAD is by converting existing biogas plants by adding an additional (hydrogen-producing) bioreactor. This would avoid the construction of new plants, a factor that significantly increases implementation costs.

In the future, genetic engineering, bioaugmentation, and AI-based algorithms could enhance the process performance and stability of TSAD. The implementation of TSAD can foster growth in different industries.

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