TWO-STAGE ANAEROBIC DIGESTION OF ORGANIC WASTES: A REVIEW

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Abstract. Anaerobic digestion is a biotechnological process, in which microorganisms degrade the 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 communities of microorganisms (acidogenic and methanogenic) taking part in the anaerobic digestion differ in many aspects e.g. their optimal conditions for cell growth and development. Therefore, the anaerobic digestion in a single bioreactor (BR) (single-phase process) required selected optimal conditions, taking into account the slow-growing methanogens at the expense of fast-growing acidogens, which affects the efficiency of the whole process. This has led in recent years to development of two-stage anaerobic digestion, in which the processes are divided into a cascade of two separate BRs. It is known that this separation 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.

The present paper aims to review the literature in the field of two-stage anaerobic digestion of organic wastes.

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

I. INTRODUCTION

Anaerobic digestion (AD) of organic wastes is a very attractive biotechnology during last years mainly in the field of the renewable energy sources and biofuels. This biotechnology is very useful for depollution of highly polluted with organics wasted waters and municipal wastes. However, using a variety of wastes and residuals as substrate and mixed cultures in the bioreactor makes AD as one of the most complicated biochemical processes employing hydrolytic, acidogenic, hydrogen-producing, acetate-forming bacteria as well as acetoclastic and hydrogenoclastic methanogens. Hydrogen (H_2) and 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 H_2 and therefore biogas plants can become biorefineries [Wainaina et al., 2019]

Certain organic wastes, however, cannot be easily digested due to their low nutrient level insufficient for AD, thus anaerobic co-digestion (AcoD) (digestion of mixtures of organic wastes) is a viable option. Numerous studies have shown that using co-substrates in AD systems improve methane yields as positive synergisms are established in the digestion medium, and the supply of missing nutrients are introduced by co-substrates. Synergistic effects among different chemical components under the AcoD process played an important role in

improving its performance [Hubenov et al., 2015; Chow et al., 2020; Zhang et al., 2021].

AD is a multi-step biotechnological process with H_2 as a non-accumulating intermediate product [Ruggeri, et al, 2015; Achinasb and Jan Willem, 2017]. Recently, the interest in H_2 production through AD, has increased [Ausiello. et al., 2017; Tashyrev, et al., 2018; Bakonyi et al., 2019; Hovorukha et al., 2021]. 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 [Gerardi, 2003]. 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 hydrogenic process followed by methanogenic process has been suggested [Demirer and Chen, 2005; Chu et al, 2012; Ruggeri, et al, 2015]. In the TSAD system (Fig. 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

resulted in significantly higher degree for the two-stage H_2+CH_4 system, as compared to the traditional one-stage CH_4 production process [Ruggeri, et al, 2015].

In our laboratory experimental studies of co-digestion of mixtures of milk, whey and wasted

activated sludge from the Sofia waste waters treatment plant in two-stage process we obtained degree of biodegradation (DBD) of 95 % [Simeonov, 2005]. In the case of two-stage processes with production of methane only, the obtained digestate may be used as a natural fertilizer in agriculture.

Fig. 1. Scheme of two-phase process of AD with simultaneous production of H_2 and CH_4 .

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 BR_1 and BR_2 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 (1):

$$D_1 = F/V_1, D_2 = F/V_2,$$

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

It is known that the V_2 of the BR_2 for methane production is larger than the volume V_1 of the BR_1 . Therefore, the constant $\gamma < 1$ should be valid.

The TSAD process is based on the different function between acidogens and methanogens in physiology, nutrition needs, growth kinetics, and sensitivity to environmental conditions. In the first stage, the substrate is transformed to H_2 , CO_2 , VFAs, lactic acid and alcohols by acidogens with optimal pH of 5.0–6.0 and hydraulic retention time (HRT) of 1–3 days. In the second stage, the remaining VFAs, lactic acid, and alcohols in the BR producing H_2 effluent are converted to CH_4 and CO_2 by methanogens under optimal pH range of 7.0–8.0 and HRT of 10–15 days.

The bottle-neck problems occur in the first stage (production of H_2) of the TSAD. In this process 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 refuses [Rafieenia Ret al., 2018]. Experimental results show that the energy produced as H_2 and CH_4 gases is higher than the harvested energy using a one-step AD; specifically it is in the range of 1.5–2.7 times higher [Ruggeri, et al, 2015].

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 with high potential to be used as vehicle fuel [Ruggeri et al., 2015; O-Thong et al., 2018].

In the early publications concerning TSAD processes production of methane only in both bioreactors is observed (old approach) [Simeonov, 2005; Parawira et al., 2007], while in the recent publications co-production of H_2 (in the BR_1) and CH_4 (in the BR_2) is available (new approach).

Some review papers in this field are known. In [Sompong O-Thong, et al., 2018] the first review of TSAD is presented. On the base of 132 references the general approach of biohythane production via two-stage AD (principles, microorganisms, reactor configuration, process parameters, methods for improving productivity as well as technical challenges toward the scale-up process of biohythane process)

are outlined. In [Nabaterega et al., 2021-137 references] a review on TSAD options for optimizing municipal wastewater sludge treatment process is presented. A multi-criteria analysis performed on 51 data points obtained from the literature showed the thermophilic-mesophilic (T-M) two-stage AD as the best option for overall % improvement in volatile solids removal and CH₄ yield over the control among the six two-stage configurations. Additionally, T-M also achieves higher pathogen reduction and better dewaterability. This article provides a guide for the implementation and practical applicability of the two-stage AD process for wastewater treatment plants. In [D'Silva et al., 2021 - 174 references] a review on microbial biogas enrichment possibilities are presented on focus as a CO₂ utilization technique, which converts CO₂ into CH₄ through specific H₂ utilizing microbial species. It was outlined that the TSAD strategy could be the best strategy able to upgrade reliably the existing AD systems to H₂ assisted pathways. In [Cremonez et al., 2021 – 205 references] a review evaluating the scenario and viability of the multi-stage AD process applied to agroindustrial effluents is presented. It is outlined that the great challenges are focused on the stability of the composition and yield of H₂ in the acid phase, besides the problems resulting from the treatment of complex residues. 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. 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 [Rabii et al., 2019 – 131 references] a review of anaerobic co-digestion including the effect of TSAD processes is presented. In [Wei and Liu, 2018 – 27 references] it is outlined that at present, TSAD technology is in its testing stage and according to some researches it is not suitable for some kinds of waste processing. In [Khan et al., 2016 – 100 references] a review that combines the optimization approaches for methane, VFAs and biohydrogen production from the AD is presented. In this review study, the types and configurations of the bioreactor are discussed for each type of product. It is outlined that by adapting the same anaerobic system for VFAs, biohydrogen and methane individually or simultaneously it could be significantly improved the economic and

environmental sustainability. However these reviews discussed some particular cases.

The recent survey presents the state of the art (on the base of 163 references, from 2002 to 2022 and our own studies), including the influence of different parameters, types of bioreactors, microbiology aspects, mathematical modelling and energetical considerations on TSAD processes

II. FEEDSTOCKS (SUBSTRATES)

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

1. Activated sludge (AD and AcoD) [Chow et al., 2020; Zhang et al., 2021; Athanasoulia et al., 2012; Simeonov, 2005; Watts et al., 2006; Zhu and Béland, 2006; Shia et al., 2021; Sun et al., 2022; Forouzanmehr et al., 2021];
2. Municipal wastewaters [Nabaterega et al., 2021; Li and Yu 2016;];
3. Organic fraction of municipal solid wastes [Bakonyi et al., 2019; Kumar and Samadder, 2020; Lavagnolo et al., 2018; Rocamora, et al., 2020; Ding et al., 2021; Nguyen et al., 2021];
4. Industrial wastes [Ramadhani et al., 2021; Krishnan et al., 2016; Fuentes et al., 2021; Kamyab and Zilouei; 2021];
5. Agroindustrial waste - milk whey, sugarcane vinasse, manipueira, vinasse, tequila vinasses, highly concentrated winery effluents, dairy manure, dairy wastewater, abattoir wastewater [Aruna Devi et al., 2018; Cremonez et al., 2021; Buback dos Santos et al., 2021; Ramos et al., 2021; Demirer and Chen, 2005; Guo et al., 2010; Rodriguez et al., 2013; Hailu et al., 2021; Vital-Jacome and Buitron, 2021];
6. Sugarbeet [Hussy, et al., 2005];
7. Cassava [Okudoh et al., 2014];
8. Grass and maize silage [Nizami and Murphy, 2011; Illi et al., 2021];
9. Organic solids waste [Kumar et al., 2020; Lin and Lay, 2003. Chu and Wang, 2017; Khalid, et al., 2011];
10. Palm oil mill effluent [Krishnan et al., 2016; O-Thong et al., 2008 ; Ramadhani et al., 2021; Sani et al., 2021];
11. Food waste and food wastewater [Ariunbaatar al., 2015; Chatterjee and Mazumder, 2018; Bolzonella et al., Yun, et al., 2018; Pramanik et al., 2019; Meena et al., 2020; Srisowmeya et al., 2020; Chu et al., 2012; Fenghao et al., 2011; Komiliset al., 2017; Pramaniket al., 2019; Seung et al., 2010; Srisowmeya, et al., 2020; Wang and You-cai, 2009;

Xiaoa et al., 2018; Hou et al., 2021a; Hou et al., 2021b; Hassan et al., 2021; Rusin et al., 2021];

12. Organic market waste [Gómez et al., 2019];

13. Kitchen waste [Jayalakshmi, et al., 2009; Yan et al., 2021 - waste cooking oil];

14. Fruit and vegetable waste [Parawira et al., 2007; Ganesh et al., 2014; Chatterjee and Mazumder, 2018];

15. Pharmaceutically active compounds [Gallardo-Altamirano et al., 2021];

16. Residual fermented solids obtained after biodiesel production [dos Santos et al., 2021]. The reported process offers the possibility of integration among three biofuels of industrial interest (biodiesel, bio-hydrogen, and biomethane), according to the biorefinery principles, which target the maximum utilization of biomass to produce a variety of products.

17. Mixtures (co-digestion) of organic waste [Baldi et al., 2019; Esposito et al., 2012; Hubenov et al., 2015; Li and Fang, 2007; Martinez-Burgos et al., 2021; Silva Fabrícia et al., 2018; Notodarmojo et al., 2022; Tena et al., 2021; Tsigkou et al., 2021; Chen et al., 2021 - swine manure and rice straw; Hailu et al., 2021 - abattoir wastewater, heterogeneous fruits-vegetables solid waste, and their combination; Yan et al., 2021 - waste cooking oil and sewage sludge, Náthia-Neves et al., 2018 - restaurant food waste and vinasse, a waste from sugarcane industry].

All authors concluded that the chemical nature of the substrate greatly influences the process and the pH optimum 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 (Rasapoor et al., 2020).

Not only the quality but also the amount of solids in the substrates submitted to digestion affects considerably the whole process (Elbeshbishy et al., 2017). 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% and solid digestion, with levels above 15% (Liu et al., 2016). Komilis et al. (2017) also classify the process in wet (TS of the raw material $\leq 10\%$), semi-dry (TS of the raw material 10–20%), and dry (TS of the raw material $\geq 20\%$) digestion.

In general, liquid digestion systems present higher intensity reactions with substrate, short 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 (Kainthola et al., 2019).

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 a high content of water and sugar being well suited as substrates for fermentation. In recent years an interesting substrate for TSAD process are lignocelluloses in organic waste [Yang, et al., 2015]

III. REACTORS CONFIGURATION FOR BIOHYTHANE PRODUCTION

Different BRs were used as H_2 or CH_4 production reactors in TSAD. More than 95% of the working industrial biogas plants operate with CSTRs. However, a lot of studies are dedicated to new type of bioreactors as follows:

- 1) Biofilm reactors [Mohan et al., 2008];
- 2) Membrane BRs [Chaikasem et al., 2014];
- 3) Upflow Anaerobic Sludge Blanket (UASB) reactors [Aruna Devi et al., 2018; Nizami and Murphy, 2011]; fixed-bed up-flow BRs [Rodriguez et al., 2013];
- 4) Anaerobic fluidized bed reactor (AFBR) [Ramos et al., 2021; Ramadhani et al. 2021];
- 5) Solid-state anaerobic BRs [Ding et al., 2021 - first-stage solid-state digester and a second-stage liquid digester];
- 6) Anaerobic sequence batch reactors (ASBR) [Hailu et al., 2021];
- 7) Combinations:
 - a) BR1 - UASB, BR2 - CSTR [Krishnan et al., 2016];
 - b) BR1 – CSTR, BR2 - anaerobic sequencing batch reactor (ASBR) and UASB [Sani et al., 2021];
 - c) BR1 – CSTR, BR2 – UASB [Kamyab and Zilouei; 2021];
 - d) BR1 – UASB, BR2 CSTR [Sposob et al., 2021];
 - e) BR1 - solid-bed reactor, BR2 -UASB [Parawira et al., 2007].

IV. TEMPERATURE AND pH

4.1. 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. H₂-producing dark fermentation reactors can be operated in various temperature ranges from mesophilic (35–45°C), thermophilic (55–60°C) to extreme thermophilic (70–80°C) conditions. Most of the H₂ dark fermentation studies have been conducted at temperature range of 35–45°C.

Temperature is also a very important operation factor in the second stage for anaerobic digestion process. It determines the rate of anaerobic digestion process, particularly the rate of hydrolysis and methanogenesis. The thermophilic process could accelerate the biochemical reactions and give higher degradation efficiency as well as higher CH₄ production rates compared to mesophilic condition [Watts and Keller, 2006]. As temperature increases, the rate of retention time process is much faster and this results in more efficient operation and lowers the retention time requirement [Van Niel et al., 2003]. Thermophilic condition also increases in thermodynamic favorability of CH₄-producing reactions, decreases solubility of CH₄ and CO₂, and destruction of pathogens in the reactor effluent. Methanogens are extremely subtle to change in temperature and even a small temperature variation (2–3°C) can lead to VFAs accumulation. This decreases the CH₄ production rate for methanogens, especially at the thermophilic conditions. Maintaining the stable temperature is important for biohythane production.

The AD process may be classified according to temperature, as [Divya et al., 2015; Kumar et al., 2020]:

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

Mesophilic bands are interesting because they make up the average temperature of most tropical countries. In addition, they ensure greater process stability and greater diversity of active anaerobic microorganisms [Kainthola et al., 2019]. Processes conducted in this temperature range are widely used when high methane levels are required, either from the process as a whole or from the digestion phase. 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 (Kainthola et al., 2019). Besides the fact that high temperature limits the amount 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

(Kainthola et al., 2019). Saratale et al., 2018) stated that the sharp increase in temperature results in an increase in the mortality rate of methanogenic organisms compared to the mesophilic phase temperatures. On the other hand, the same conditions also considerably reduced the concentration of pathogens harmful to the process (Panigrahi and Dubey, 2019), 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 Archaeas in the methane production phase (Wang et al., 2018; Chen and CHANG, 2020; LI et al., 2020b). A similar condition was also reported in a study conducted by Ruffino et al. (2020), using temperatures of 50 °C for the acid phase and 38 °C for the methanogenic phase. The authors obtained an increase in DBD from 42% to 55% and an increase in methane production from 280 to 332 L/kg added organics.

Existing combination for TSAD:

- 1) Mesophilic-mesophilic [Gallardo-Altamirano et al., 2021; Nguyen et al., 2021; Shia et al., 2021; Náthia-Neves et al., 2018]
- 2) Thermophilic- mesophilic [Krishnan et al., 2016; Ramos et al., 2021; Tena et al., 2021; Chen et al., 2021].
- 3) Mesophilic-thermophilic [Gianico et al., 2014; Vital-Jacome and Buitron, 2021]
- 4) Thermophilic-Termophilic [Ramos et al., 2021; Chaikasem et al., 2014; Kovalev et al., 2021a; Kovalev et al., 2021b]
- 5) Psychrophilic-psychrophilic (21 – 1 °C) [Rusín et al., 2021];

In [Parawira et al., 2007] three sets of temperature conditions (with different types of BRs as well) were investigated: (I) mesophilic + mesophilic, (II) mesophilic + thermophilic and (III) thermophilic + thermophilic with different optimal results concerning the yields of methane and DBD.

4.2. pH and alkalinity

Among all the chemical factors influencing dark fermentation, the pH is considered the most essential. It influences the stability of the acidproducing fermentative bacteria and acetoclastic CH₄-producing archaea. Ph plays a major role in the oxidation-reduction potential of the anaerobic process. Thus, it directly impacts the metabolic pathway. In most of literature reports, a pH of 5.5

has been considered to be the optimum pH for H₂ production [Hawkes et al., 2002; O-Thong et al., 2008; Hawkes et al., 2007; Cai et al., 2010]. The optimal initial pH range for the maximum H₂ yield or specific H₂ production rate is between pH 5.5 and 6.5 [Cai et al., 2010]. The optimal pH is highly dependent on the microorganisms. The control of pH and alkalinity of a substrate is essential for first stage dark fermentation since organic acids produced tend to decrease the pH. The pH lower than 4.5 tends to inhibit the activity of hydrogenases. Low pH also causes a shift of metabolic pathways of dark fermentation microorganisms away from H₂ production. H₂-producing bacteria like *Clostridium acetobutylicum* can change metabolism from H₂ (acetate and butyrate pathway) to the production of solvents (acetone and butanol pathway) when the pH is decreased to less than 5.0 %. Alternatively, depending on the organism, low pH can shift the metabolism toward ethanol production [Li and Fang, 2007]. CH₄ production is favored at alkaline pH exhibiting maximum activity at pH of 7.8–8.2 [Deublein, and Steinhauser, 2008].

The rate of CH₄ production may decrease if the pH is lower than this optimal range. The pH is also an important factor for the stability of CH₄ production. The H₂ effluent which is rich in VFAs, may cause a drop in pH if fed with high OLR. The pH adjustment can be achieved by an addition of alkali chemical, typically calcium carbonate or sodium hydroxide. A cheap material like ash was used to adjust the pH in an anaerobic reactor [Lo et al., 2009].

V. ANOTHER PROCESS PARAMETERS AFFECTING BIOHYTHANE PRODUCTION

5.1. Inoculums

The choice of the 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 [Jeihanipour et al., 2011] and may considerably reduce the lag phase time (Yang et al., 2015), especially in more complex systems. Ma et al. (2021) reviewed the literature seeking to quantitatively correlate the production of methane with the abundance of microbiota in anaerobic digestion processes.

Developing an enriched inoculum is very important for obtaining H₂ in first stage fermentation. In the enrichment process, selection procedure was

applied to selectively promote H₂-producing bacteria and eliminate H₂ consumers.

5.2. Hydrogen partial pressure

The H₂ partial pressure in the liquid phase is the major factor affecting H₂ production, as high H₂ partial pressure causes deactivation of hydrogenase enzyme.

5.3. Hydraulic retention time (HRT)

The total time that cells and soluble nutrients reside in the reactor is called the HRT. H₂ production occurring at low HRT is dependent on the volume of the reactor and the flow rate of feed. It is generally well known that the H₂-producing bacteria are fast growing. By applying this principle, [Liu et al., 2019] produced H₂ free of CH₄ in continuously CSTR feeding with household solid waste at acidic pH range of 5.0–5.5 and a short HRT of 3 days ($D = 0.33 \text{ day}^{-1}$) without any pretreatment to inhibit methanogens contained in the initial digested manure.

HRT is the main optimization parameters of continuous H₂ dark fermentation bioprocesses. In the CSTRs, short HRTs or high dilution (D) rates can be used to eliminate methanogens, which have significant low growth rate [Hawkes et al., 2007; Kyazze et al., 2007]. However, HRT is needed to be maintained in a proper level that still gives a D value less than specific growth rate of H₂-producing bacteria. Generally, short HRT is considered to favor the H₂ fermentation metabolism [Hawkes, 2002]. On the other hand, too high loading rates may result in substrate inhibition effects, improper food to microorganism (F/M) ratios of H₂ producers or washout of microorganisms [Lay, 2001].

In second stage, the HRT is a measure to describe the average time that a certain substrate resides in a digester. If the HRT is shorter, the system will fail due to washout of microorganisms. HRT for anaerobic digestion process are typically in the range of 15–30 days at mesophilic conditions and 10–20 days at thermophilic conditions [Mamimin et al., 2015].

5.4. C/N ratio

The carbon found in the 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 (Weiland, 2006; Khalid et al., 2011). 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 (Esposito et al., 2012; Rocamora et al., 2020). Both conditions are detrimental to biogas production. Mixing substrates of different properties may help in equalizing these essential elements. In addition, a good control and pH stability might ensure a reduction in ammonia toxicity in the reactors (Yao et al., 2020).

5.5. Trace elements

Biohydrogen and biomethane production required various types of metal ions as micro-nutrients. These metal ions play a critical role in the metabolism of microorganisms. Metal ions such as Fe^{2+} , Zn^{2+} , Ni^{2+} , Na^+ , Mg^{2+} , and Co^{2+} play a pivotal role in both biohydrogen and biomethane process. Metals are essential to supplement in media for dark fermentation. These micronutrients might be required in trace amounts but they have an influential role as cofactors, transport processes facilitators, and structural skeletons of many enzymes (Fe-Fe hydrogenase and Ni-Fe hydrogenase) involved in the biochemistry of H_2 formation [Romero-Guiza, 2016]. Therefore, several researchers have studied the effect of supplementation of Fe ion on biohydrogen production. [Hussy et al., 2005] performed 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. 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 that most of the enzymes involved. Parameters Affecting HPB Activity 19 in hydrogen production contain iron-sulfur clusters, as previously described; the increase in sulfate at 3,000 mg SO_4^{2-} L⁻¹ inhibits the production of hydrogen, due to a change in the metabolic pathway towards butyrate and ethanol.

5.6. 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 [Kovalev A.A. et al., 2021].

Our own studies concern TSAD of corn steep liquor (CSL) with simultaneous H_2 and CH_4 production in pilot scale biogas plant with automatic control system [Chorukova et al., 2021]. As a substrate in the AD process with hydrogen production used CSL – a waste product from the process of processing corn grain to extract starch. The corn extract was provided by “ADM Razgrad EAD” (Bulgaria).

All experiments were conducted in two connected bioreactors. First one with work volume of 10 dm³ (for H_2 production), second one with 80 dm³ (for CH_4 production), the ratio $V_2/V_1=10$. Initial experiments as well as inoculum maintenance was carried out in two laboratory scale bioreactors each one with work volume of 1 dm³ and 2 dm³. In all experiments mesophilic temperature was maintained (35°C) and continuous stirring mode. As an inoculum the liquid fraction from anaerobic digester operating at mesophilic conditions was used.

VI. MICROBIOLOGY

The microbiome residing in anaerobic digesters drives the AD process to convert various feedstocks to biogas as a renewable source of energy. This microbiome has been investigated in numerous studies in the last century [Lim et al., 2020; Hailu et al., 2021; Gallardo-Altamirano et al., 2021; Shia et al., 2021; Sposob et al., 2021]. 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, the rate-limiting step inherent of these feedstocks. To achieve efficient AD, a diverse microbiome is required [Hassa et al., 2018]. The anaerobic digestion 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 [Liu et al., 2008]. 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 [Kotsyurbenko, 2005].

When involving cellulose-containing substrates, the species that degrade cellulose belong mainly to the genera *Cytophaga*, *Cellulomonas*, *Cellvibrio*, *Bacillus*, *Clostridium* and *Sporocytophaga* [Szostak-Kotowa, 2004]. The strictly anaerobic, thermophilic bacterium *Ruminiclostridium thermocellum* is the microorganism with the fastest documented growth rate on the recalcitrant substrate crystalline cellulose [Lynd et al., 2002]. These organisms achieve a remarkable ability by forming 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* [Bayer et al., 2008]. Molecular biology techniques overcame the limitations of cultivation-based methods and allowed the identification of unculturable microorganisms, revealing the high diversity of microorganisms involved in AD. Several methods had been applied to investigate this microbial diversity, where in recent years metagenomics appears most efficient for determining the complex microbiota structure. In recent studies of AD processes was proved that the degradation is most probably due to the presence of members of genera *Clostridium*, *Bacteroides* and *Ruminiclostridium*. Among them, the most spread species are *Clostridium butyricum*, *Bacteroides oleiciplenus* and *Ruminiclostridium papyrosolvens* [Najdenski et al., 2020]. According to Wang et al., 2016 [Wang, et al., 2016] *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were the most abundant phyla, which were over 60% of the total relative abundance in two-phase systems and over 40% in single-phase, while all detected *Archaea* belonged to class *Methanomicrobia* of the phylum *Euryarchaeota*, which were known as methanogenic microorganisms. In many studies the microbial community analysis provided crucial information to understand the anaerobic digestion process which may help to improve its efficiency [Rivière et al., 2009; Maspolim, et al., 2015; Xu, et al., 2018; Gao, et al., 2021].

The TSAD process is based on the differences between acidogens and methanogens in physiology, nutrition needs, growth kinetics, and sensitivity to environmental conditions. The acidogens and methanogens are enriched separately in two tanks enabling optimized growth by maintaining proper environmental conditions in each reactor [Demirel and Yenigün, 2002]. Microorganisms involved in the first stage H_2 production and in the second stage CH_4 production via two-stage anaerobic fermentation process are shown in Table 1.

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 differences currently known between HCB and HPB concern their resistance to temperature, the extreme environmental conditions and the different growth rate of each species [Ruggeri, et al, 2015]. Bacteria belonging to the genus *Clostridium* are the main ones responsible for H_2 production. They are obligate anaerobes, Gram-positive and rod-shaped. *Clostridium sp.* have a substantial characteristic that distinguishes them from other bacteria allowing the production of biohydrogen in anaerobic processes instead of biomethan: 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 [Brock et al., 1994]. It happens in a specific case, in fact, that when the *Clostridium* spores are placed in favorable conditions, with nutrients and anaerobic conditions, the germination and metabolism processes can restart [Stanier et al., 1986], and consequently hydrogen and other metabolic products can be produced.

Table 1. Microorganisms involved in the first stage H₂ production, and the second stage CH₄ production via two-stage anaerobic fermentation process [O-Thong et al., 2018]

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

Enterobacter sp. are also H₂-producing microorganisms with the advantage that they are facultative bacteria able to grow in the presence of oxygen. Based on phylogenetic analysis of the rDNA sequences, Fang et al. 2002 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 [Oremland, 1988]. 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 hydrogenproducing seeds [Zhu et al., 2006].

The following list summarizes the main differences between HPB and HCB:

- Most methanogens are limited to a relatively narrow pH range (about 7–8) [Oremland, 1988], while most HPB can grow over a broader pH range (4.5–7) [Zhu et al., 2006].

- 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.

In our own research several methods had been applied to investigate their microbial diversity: clone library of 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE) analysis, fluorescence in situ hybridization (FISH), etc. Metagenomics is an efficient method for determining the complex microbiota structure and performing metabolic mechanism analysis. It is applied for elucidation of community structure and metabolic pathway analysis to determine the mechanism of cellulose degradation in natural consortia.

VII. MATHEMATICAL MODELLING

A lot of models describing separately the fermentative H₂ production [Nasr et al., 2013; Wang and Wan, 2009, 34, 3313-23] and the AD for CH₄ production [Batstone et al., 2002; Dochain and Vanrolleghem, 2001, Wade, 2020] are known. However, only few models of the TSAD process are known from the available literature [Blumensaat and Keller, 2005; Rodriguez et al., 2013; Donoso-Bravo et al., 2019; Kamyab and Zilouei, 2021].

Our team developed mathematical models of the TSAD processes started from simple models [Simeonov and Chorukova, 2016; Chorukova et al., 2016; Diop et al., 2017; Chorukova and Simeonov, 2020]. They are useful for software sensors and automatic control algorithms design.

Some of our models were developed on the base of the well known mathematical model of IWA ADM1 [Borisov et al., 2016; Borisov et al. (a), 2020; Borisov et al. (b), 2020]. 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 by simulations (Fig. 2, Fig. 3).

Fig. 2. Input-output static characteristic $Q_{H_2} = f(D_1)$ of BR_1 .

Fig. 3. Input-output static characteristic $Q_{CH_4} = f(D_2)$ of BR_2 .

From Fig. 2 and Fig. 3 is evident that a maximum of biogas yield exists for both BRs (for biohydrogen and for biomethane). That is the base for application of the so called “extremum seeking control”.

In the known literature more of the TSAD processes are operated manually and the ratio of working volume of CSTR bioreactors is not discussed. In some reports a ratio of 10 is accepted without comments [Gómez Camacho et al., 2019]. According to Dareioti and Kornaros [Dareioti et al., 2014] a TSAD system consisting of two CSTRs operating at mesophilic conditions was used to investigate the effect of hydraulic retention time

(the reciprocal value of the dilution rate) on H_2 and CH_4 production. The ratio of working volumes of bioreactors is equal of 8 (without comments). Cavinato et al. [Cavinato et al., 2011] performed optimization of TSAD with separately collected municipality biowaste in a pilot scale CSTRs at thermophilic regime using recirculation of the digestate of the second BR to maintain the pH in the BR_1 at the optimal value. The ratio of working volumes of bioreactors is equal of 3.8. Optimal loading rate is obtained providing maximum H_2 and CH_4 productions in TSAD of cassava wastewaters using specific thermophilic bioreactors and constant recycling ratio of 1:1

with automatic control of pH in the hydrogenic bioreactor in the report [Intanoo et al., 2014]. The ratio of working volumes of both bioreactors is equal of 6 (without explanation). In the work [Schievano et al., 2014] both bio-hydrogen and bio-methane productions are optimised in thermophilic bioreactors regarding two operating parameters (organic loading rate and dilution rate), however, the first bioreactor is running in semi-continuous and the second - in batch operation mode, because working volumes of both bioreactors are equal. The first review article [Khan et al., 2016] combines the optimization approaches for three possible products from AD - H_2 , CH_4 , and VFAs, taking into account different process parameters and types of BR, including acidogenesis and methanogenesis separation in two different BRs. However, the ratio of working volumes of the BRs is not included and discussed.

In the TSAD processes an important from technological and economical point of view problem exists – how do determine the ratio of the working volumes of the H_2 and CH_4 producing BRs for optimization of all processes. In [Buback dos Santos et al., 2021; Ganesh et al., 2014] possible solutions of this problem are presented in view of maximizing the energy production (the produced H_2 and CH_4). Our team [Chorukova et al., 2021] proposed a solution of

this problem for CSTR on the base of the developed mathematical models.

VIII. ENERGETICAL 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 [Nsair, et al., 2020]. This also applies to biogas as a source of renewable energy, where the number of biogas plants installed in Europe has been increased from 6227 in 2009 to reach 18202 by the end of 2018 [Sevillano et al., 2021]. The total produced electricity from biogas reached 88 TWh in 2017, 40% of which was generated in Germany [Sevillano et al., 2021]. Hence, Germany is a leading country in this field. Due to more than 9000 large-scale AD plants, biogas technology is making a significant contribution to the sustainable energy supply in Germany [Weinrich and Nelles, 2021]. 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 accounts for over 58% of total electricity generation from biomass. In Germany, anaerobic digestion plants usually use renewable raw materials and animal excrements (manure and dung) to operate.

Our experimental results are summarized from energetical point of view in Table 2 [Simeonov et al., 2021].

Table 2. Experimental results obtained during the TFAD continuous tests ($\gamma = V_1/V_2 = 0.1$ and $S_{0i} = 50$ g/L).

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 TFAD of corn steep liquor are in the initial phase of technology development and were at low organic loads on both BRs.

Other representative results [Ruggeri, et al., 2015] for TFAD of organic wastes are presented in Table 3, where the operative conditions for the

continuous tests, the specific H_2 and CH_4 production (mean value), the total energy harvest by TFAD and the efficiency values are given.

The efficiency can be evaluated as: $\eta = \text{Produced energy } (H_2 + CH_4) / \text{Initial energy embedded in the substrate} \times 100$ (%).

Table 3. Experimental conditions and results for the TFAD continuous tests ($\gamma=0.1$).

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
H ₂ production (L L ⁻¹ day ⁻¹)	1.67	0.49	0.19
CH ₄ 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

IX. CONCLUSIONS

Methane is being commonly used, not only in the chemical industry but also in transport as compressed natural gas, which has been regarded as the clean energy carrier in comparison to gasoline or diesel. However, by combining the advantages of H₂ and CH₄, biohythane is considered one of the important fuels involved in achieving the transition of technical models from a fossil fuel-based society to renewable-based society. In addition to economical concern, the TSAD process has been previously evaluated that the payback time is around 2–6 years, depending on the disposal costs of organic wastes/residues [Wang and Zhao, 2009].

Various types of organic wastes can be used as substrate for biohythane production.

Biohythane via TSAD of organic waste could be a promising technology for higher energy recovery and a cleaner transport biofuel than the biogas. The H₂/CH₄ ratio of range 0.1–0.25 is suggested for biohythane.

The TSAD could increase DBD, net energy balance, CH₄ production rates as well as high yield and purity of the products. In addition, the TSAD process has advantages of improving negative impacts of inhibitive compounds in feedstock, increased reactor stability with better control of the acid production, higher organic loading rates operation, and significantly reducing the processes time.

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