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Study of a biological process for the combined production of hydrogen and methane and injection into the gas grid.

S.S.D. ICHI-02/A - Impianti chimici

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Declaration

I, Davide Bertasini, hereby declare that this thesis represents my own work and has not been submitted for another degree, either at University of Verona or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University of Verona concerning plagiarism and intellectual property.

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Abstract

The European Union is aiming for climate neutrality by 2050. And to lower emissions derived from the use of natural gas, proposed by 2030 to achieve the production of 35 bcm of biomethane from anaerobic digestion processes. An alternative version of the process has become popular in the scientific community due not only to the capacity to increase biomethane yield but also for the recovery of biohydrogen. Blending it at 2%-20% v/v with methane would preserve the ability to be injected into the gas grid for common use, thus leading to a reduction in emissions. The process is based on the use of a second upstream reactor to separate the specific parameters of acidogenic fermentation and methanogenic phase.

This thesis investigates the application of the two stage anaerobic digestion (TSAD) process in the agricultural context, specifically evaluating agricultural residues as a readily available lignocellulosic substrate. Addressing challenges for process optimisation and continuous biohydrogen production, by testing several strategies reported in the literature, such as the use of pretreatment. And, evaluating in detail the fermentation at different hydraulic retention times of livestock effluents, such as manure, and vegetal residues. Managing to achieve by plant derivatives' co-fermentation a continuous biohydrogen production, with 12.35 $NmLH_2/gVS$. Lastly, concluding with an economic evaluation of the applicability of the process, based on the results obtained.

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-**D. Bertasini**, F. Battista, F. Rizzioli, N. Frison, D. Bolzonella. Decarbonization of the European natural gas grid using hydrogen and methane biologically produced from organic waste: A critical overview, 2023. Renewable Energy 206 (10.1016/j.renene.2023.02.029).

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-F. Battista, C. Padovan, E. Salvetti, V. Gatto, F. Rizzioli, **D. Bertasini**, D. Bolzonella. A groundbreaking biorefinery loop for the valorization of cigarette butts into fermentable sugars and bioethanol, 2023. Sustainable Chemistry and Pharmacy 31(287 7):100948 (10.1016/j.scp.2022.100948)

-C. Magonara, E. Montagnese, **D. Bertasini**, C. Vona, G. Salvatori, L. Nguemna Tayou, M. Villano, F. Battista, N. Frison, D. Bolzonella, G. Pesante. Mixed culture polyhydroxyalkanoate production with variable hydroxyvalerate content from agri-food residues, 2025. Environmental Science and Pollution Research (https://doi.org/10.1007/s11356-025-36316-4)

Under review

-F. Battista, **D. Bertasini**, F. Rizzioli, E. Salvetti, D. Bolzonella, D. Slaghenaufi, M. Ugliano. Red grape marcs valorization in a biorefinery loop for the production of short and medium fatty acids, hydrogen and methane. Fuel Processing Technology.

In process

-**D. Bertasini**, F. Valentino, R. Lauri, N. Frison, D. Bolzonella, F. Battista. Optimizing specific dark fermentation strategies for enhanced Bio-Hydrogen production from different agricultural wastes.

1. Introduction

1.1. Background

In 2024 the anthropic carbon emission exceeded 37 Gt [1] contributing to the average global temperature increase of 1.45 °C since the pre-industrial era [2]. Hydrogen, never more than now, is considered a promising fuel for the future due to its zero carbon emissions, but however, 96% of hydrogen generation is still fossil-based [3]. The emerging technology of Two Stage Anaerobic Digestion (TSAD) permits the combined production of biomethane and biohydrogen with a neutral carbon impact, and furthermore, could be used to reduce organic waste disposal. The biohydrogen produced from the process can be injected 2%-20% v/v in the current gas grid (according to the regional limitations) leading to partial decarbonization [4]. In this regard, the current employment of natural gas is responsible for about 20% of the total carbon footprint. Given the European Union's imposition to achieve the digester potential of 35 bcm by 2030 [5], the thesis was focused on studying how the TSAD process could be integrated as a greener solution. In particular, agrowaste were investigated, being about 57% (0.8 EJ) [6] of feedstocks employed in AD plants, and also, constituting a disposal problem due to the huge quantity generation (about 19 Mt/y dry of vegetal and 103.2 Mt/y of manure in Italy) [7,8].

1.2. Hydrogen

Hydrogen is defined as the green fuel par excellence. In fact, its consumption produces only water as a by-product. Hydrogen is also better from an energetic point of view as it has a lower heating value (LHV) of 120 MJ/Kg, higher than 50

MJ/Kg of methane, which guarantees high performance. Hydrogen European demand reached 7.9 Mt in 2023, mainly driven by refinery (57%), chemical industries for ammonia (25%) and methanol (11%) production [9].

As previously reported, 96% of hydrogen production is still fossil-based, primarily made through natural gas steam reforming. Rarely hydrogen is produced from other technologies such as partial oxidation and autothermal reforming, involving 7-9 $KgCO_2$ released per hydrogen kilo. Only 0.2% of hydrogen is produced employing electrolysers, which count a total capacity of 162 MW, but an increase to 140 GW is expected by 2030 in conformity with the goals of the Hydrogen Strategy [10]. The electrolysers utilization shows limits due to the electrical energy availability and building materials needed, about 40 different raw materials and 60 processed (98% of them are extra Europe imported). To ensure renewable hydrogen production, the energy sources have to be sustainable, consequently, in major cases the excess electricity from solar and wind plants is used. In this regard, continuous production is not guaranteed, but only punctual, leading to correlated storage and employment difficulties [4]. Based on the origin, hydrogen is categorized into biologic (biohydrogen), or non-biologic; the latter is further subdivided for the technological processes into 11 colours. The hydrogen product of steam methane reforming is defined as Grey and as Blue with the addition of a carbon capture or reduction system, gasification of biomasses and bituminous coal is responsible for Brown and Black hydrogen production. Turquoise hydrogen results from methane pyrolysis, Red hydrogen is produced through thermochemical processes, and nuclear power used in electrolysis produces Pink hydrogen, becoming Purple Hydrogen with the combination of heat. Green hydrogen is generated from water electrolysis using renewable energy, in specific, it is recognized as Yellow hydrogen from solar energy and Orange hydrogen from different kinds of electricity of the grid. Their cost varies widely, for now fossil sources remain the cheapest option: Grey (0.77-2.52 €/KgH2), Black/Brown (1.43-2.74 €/KgH2), Blue (1.54-3.51 €/KgH2), Turquoise (1.76-3.73 €/KgH2), Red (2.41-2.85 €/KgH2), Orange (3.68-3.95 €/KgH2), Pink/Purple (3.47-6.39 €/KgH2), Yellow (8.67-9.22 €/KgH2), Green (2.09-11.94 €/KgH2) and for Biohydrogen (1.15-9.65 €/KgH2) [11–13].

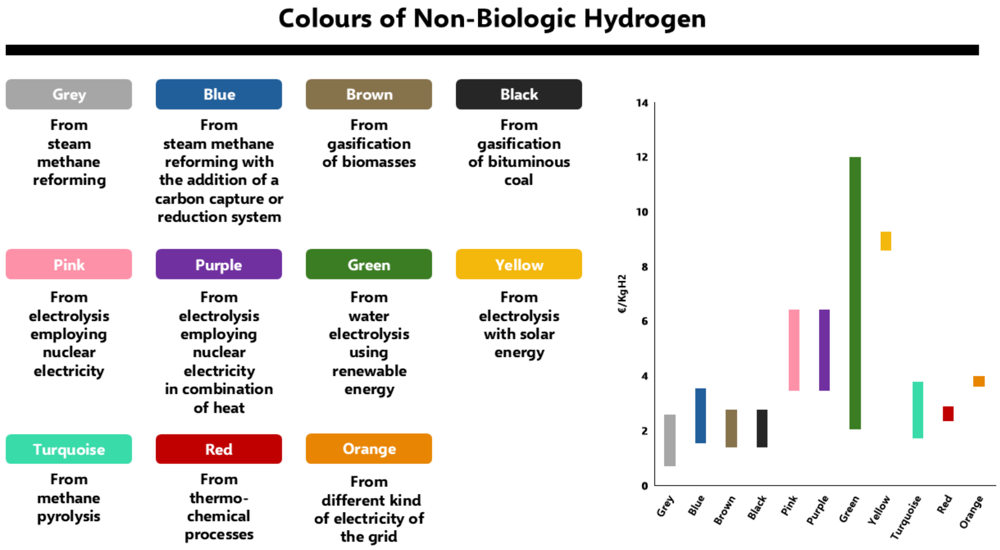

Figure 1. Colours of non-biologic hydrogen and related costs per kg

Although, institutions are expected to take charge of the cost variations to make renewable hydrogen more competitive, in view of achieving decarbonization. Nowadays, pure hydrogen is not injected into gas network, as it requires the use of a specific pipeline with a diameter of more than 0.3 m and 1.6 times thicker than current natural gas pipes. In this regard, several European projects have been funded for constituting a specific grid for hydrogen transport, such as the "European

Hydrogen Backbone Initiative" [14,15]. Moreover, it is necessary to consider the difficulty in pure hydrogen storage, due to its intrinsic high volatility and explosivity. For this problem, several solutions have been developed, such as the conversion into more stable and easily transportable compounds, such as methane (through the Biomethanation process) or ammonia. Alternatively, the employment as "biohythane" in the gas grid, i.e., a gaseous blend of biohydrogen and methane that, depending on regional restrictions, can be operated at 2%-20% v/v in H_2 volume. The replacement of natural gas with this mixture would: I. Reduce the carbon emissions during combustion; II. Improve the performances of flammability range and combustion stability; III. Reduce the hydrogen storage problems.

In this regard, anaerobic digestion (AD) permits the simultaneous generation of biomethane and biohydrogen from organic feedstocks when operating in two stages. This innovative process is called two stage anaerobic digestion and is still being studied for its potential to increase biomethane generation compared to traditional AD, and, its capacity to couple it with biohydrogen production. Additionally, TSAD can be adopted as a green disposal system, permitting to employ organic waste as a substrate, leading the process to fit into the virtuous system of circular economy.

In Paragraph 1.4., the process is described in detail.

1.3. European Hydrogen Strategy

In 2020, the European Commission (EC) established hydrogen as a key for decarbonization and adopted the "EU Hydrogen Strategy" intending to produce at least 10 Mt of renewable hydrogen and to import the same quantity by 2030 [3]. In this regard, 15 European countries included hydrogen production in their "Recovery and Resilience Plan". A strategy developed to achieve the "Green Deal" goals set in 2019: I. Zeroing the Greenhouse Gases (GHG) emissions by 2050; II. Dissociate the economic growth from the resources' usage; III. No neglect of person or place [16]. In support of the Hydrogen Strategy, the EC has issued the "Fit-For-55 Package", a series of investment funds on H_2 and related projects, and, the "REPowerEU" politic. The "Fit-For-55 Package" is a series of legislative proposes released in 2021 to meet the reduction of 55% in carbon emission, compared to the 1990 level [17]. The package is supported by the pre-existing "Renewable Energy Directive II" (RED II), which established the criteria for the definition of "renewable hydrogen", as it should ensure at least a 70% reduction in emissions [18,19]. Furthermore, EC is making funds available to accelerate the investments in hydrogen, for example, the "Recovery and Resilience Facility for Clean Energy" ([20]) and the "Important projects of common European Interest" (IPCEIs) [21]. Finally, the adoption of REPowerEU of 2022 with the goal of reducing dependence on Russian natural gas is further pushing the focus on hydrogen development for the necessity to reach a diversified energy supply [5,22,23].

Italy is among the 15 countries that have elaborated their hydrogen national strategy, in particular, incorporating it firstly in the "Strategia Energetica Nazionale" and the "Piano Nazionale integrato per l'energia e il clima" with targets by 2030. Targets that have been expanded by 2050 with the development of "Strategia a lungo termine". Italy recognizes the fundamental role of H_2 in decarbonization, and forecasts to increase its energy contribution from the current

2% to 13%-14%. The strategy planned the hydrogen employment in transport systems (trucks and trains working with fuel cells), in the industry (chemical and refinery), and injected as biohythane (i.e., mixed with methane) in gas network. In Italy, the natural gas network grid is about 70 bcm and it is responsible for 160 Mt of CO_2 emissions. The aim set is to achieve at least the 2% v/v of hydrogen injected by 2030. The strategy highlighted the institution's importance, as responsible for determining the hydrogen competitivity that will be based on the electrical energy and natural gas costs [24,25]. And the development started by focusing on "Hydrogen Valleys" realities, i.e., a narrow area where the hydrogen system is tested in the whole process of generation-transport-usage [26].

1.4. Two Stage Anaerobic Digestion process

The AD is a well-known technology that is largely employed for biogas and biomethane production, intended for co-generation, electricity production, or, in the case of the latter, injection in the gas grid. In Europe, the number of plants is above 20,800 with a total production of 21 bcm of renewable gas (4.2 of biomethane) [27]. The plants are in the major cases located close to the source of the feedstocks, such as energy crops, agrowaste, OFMSW, and wastewaters, thus minimizing the carbon impact of transporting tons of material. The biological process is commonly performed in a single-phase reactor from a mixed culture of acidogens and methanogens microorganisms, where 4 metabolic steps happen simultaneously: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. During the hydrolytic step, the particulate material is decomposed into soluble compounds. Then external bacteria enzymes attack complex compounds to reduce them to simple monomers.

In particular, carbohydrates in sugars, proteins in amino acids and peptides by proteases, and lipids in long chain fatty acids (LCFAs) by lipases. Being the slowest step of the four, its speed is essential in determining the rate of the entire process. Hydrolysis is in fact the limiting step in AD. Therefore, the type of substrate employed has a high influence on the process itself. For example, a plant operating lignocellulosic material will require long lead times for the production of biomethane and degradation of the feedstock. The second step is acidogenesis and it is responsible for volatile fatty acids (VFAs), carbon dioxide, and biohydrogen production. In detail, the acids formed are acetic acid, propionic acid, and butyric acid from sugars and amino acids, while LCFAs are responsible mainly for the acetate [28,29].

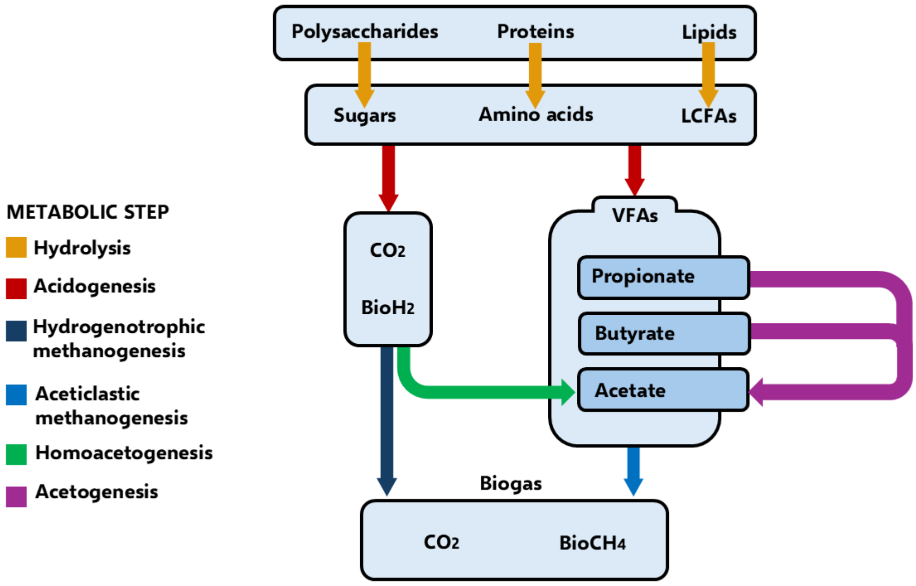

Figure 2. AD process and intermediate phases

The VFAs are short/medium chain carboxylic acids, i.e. formic acid (C1), acetic acid (C2), propionic acid (C3), butyric acid (C4), valeric acid (C5), and caproic acid

(C6). Widely employed in the industry either as raw material or as finished compound, reaching high prices on the market (between 1,800 €/ton and 2,250 €/ton) for chemicals [30], pharmaceuticals, and cosmetics production. Currently, the production is petrochemical and thus has an important environmental impact [31]. The acetogenesis is an intermediate phase involving only part of the metabolites, which is responsible for propionate and butyrate conversion in acetate [28,29,32]. In some cases (as stress conditions or lack of reduced compounds), part of acetogens can switch the metabolism to homoacetogenesis, where CO_2 and bioH_2 are consumed in acetate production [33,34]. Finally, in methanogenesis, two main pathways can occur for the conversion of metabolites into biomethane and carbon dioxide (typically 65% and 35% v/v in stable conditions). Acetoclastic methanogenesis (Equation 1) employs acetate for biomethane and carbon dioxide production. Hydrogenotrophic methanogenesis (Equation 2) is based on hydrogen and carbon dioxide reaction for biomethane production. Two other alternative versions of methanogenesis occur rarely, due to methanol presence: H_2-independent methylotrophic (Equation 3) and H_2-dependent methylotrophic methanogenesis (Equation 4) [35].

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \qquad \text{(Eq.1)}$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \text{(Eq.2)}$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \qquad \text{(Eq.3)}$$

$$CH_3COH + H_2 \rightarrow CH_4 + H_2O \qquad \text{(Eq.4)}$$

The final solid-liquid product is called digestate and it is enriched in nutrient as nitrogen and phosphorus. Digestate is often employed as a soil conditioner, alternatively is treated to recover nutrients and produce fertilizers.

The AD process in the presence of sulfate can house also the sulfate-reducing bacteria, microorganisms able to convert it into hydrogen sulfide. Hydrogen sulfide is toxic to methanogens and can cause inhibition phenomena (> 0.2 gH_2S/L). In particular, there exist two types of sulfate-reducing bacteria: type I, as *Desulfovibrio*, oxidates acetate, while type II, as *Desulfobacter*, employs fatty acids. A possible solution is to add in the reactor iron compounds to precipitate the hydrogen sulfide as iron sulfide [29].

The anaerobic digestion process is defined based on the total solids (TS) content as dry (20%-40% TS w/w) and wet (≤ 15% w/w), and the operating parameters applied depend on the setup and are within the ranges shown in Table 1 below. Typically, reactors are operated at mesophilic temperatures, as these ensure good process stability and the achievement of satisfactory methane yields. Psychrophilic conditions, on the other hand, allow lower energy expenditure for maintaining the plant, but hardly achieve high yields. Lastly, thermophilic conditions, although with certain substrates guarantee higher yields than mesophilic conditions. Maintaining stability is more arduous and, in addition, has a strong impact on costs, especially when the temperature range between inside and outside the chamber is significant. The correct C/N ratio is crucial for the operation of the reactor as it ensures the presence of the macro-nutrients essential to the microbial community, as carbon and nitrogen. If the ratio is too high, there is a risk of nitrogen deficiency, an element essential for protein synthesis and the functioning of cell metabolisms. Conversely, a ratio that is too low, could highlight a deficiency in carbohydrates, the primary source of energy for the cell. At the same time, high nitrogen contents could be synonymous of ammonia inhibition (over 3 g/L) [29]. Depending on the reactor volume, a high organic loading rate (OLR) and a low hydraulic retention time (HRT) can be negative for the process, causing washout or pH inhibition phenomena. In the first case, the feedstock flow is too elevated and the replication and growth rate of the microbial community cannot keep up, leading to the emptying of the microbial part. For the second case, this setup favors the acidogenic fermentation leading to high VFAs production. An accumulation of acids can decrease the pH of the medium below the methanogenic range, inhibiting the process. The employment of low OLR and high HRT facilities the organic matter degradation and the biogas production, 60-90 days are estimated for the complete conversion of polymers [36]. Simultaneously, this setup requires the use of reactors with very high volumes and the maintenance of operating temperatures for several days. This therefore implies a greater impact on implementation and operating costs. In addition, based on the substrate, a low OLR can determine nutrient scarcity for the microbial community, leading the process to inefficiency [37]. For each plant, it is, therefore, necessary to balance the operating parameters in order to optimize the process without burdening costs.

Table 1. AD operative parameters, in terms of temperature, pH, C/N ratio, OLR and HRT.

		Anaerobic digestion
Temperature	°C	10-30 °C (Psychrophilic)
		30-40 °C (Mesophilic)
		50-60 °C (Thermophilic)
pH	-	6.8-8.2
C/N	-	20-40
OLR	$\frac{kgVS}{(m^3 \times d)}$	1.2-12.0
HRT	d	>20

References [29,36,37]

The process can be performed in continuous or discontinuous mode, and there are several reactor configurations used depending on the substrate. The most commons are Anaerobic Plug Flow Reactors (APFR), Continuous Stirred Tank Reactors (CSTR), Up-flow Anaerobic Sludge Blanket (UASB) and Fixed Film Reactors (FFR) [37].

Once biogas is produced, it can be used for co-generation, but cannot be fed into the gas grid as it is, as it still contains traces of contaminating compounds (such as CO_2, water vapor, ammonia, and H_2S). Therefore, a cleaning and “upgrading” step is required to remove these compounds and obtain biomethane (up to 99% v/v). Commonly, this process takes place on-site and involves the use of a membrane separation process or adsorption methods such as water or organic solvent scrubbing [27,36].

The alternative version TSAD, physically separates in two chambers the Dark Fermentation (DF) (the first two metabolic steps) from the methanogenic phase. This separation permits to improve the two reactions, adopting specific operational parameters. In particular, low HRT and high OLR favor acidogens, known for their fast-growing and fast-reproducing kinetics (from hours to few days), as *Firmicutes* (genera *Enterococcus*, *Clostridium*, *Bacillus*, *Prevotella*, *Escherichia*). While high HRT and low OLR are preferred by methanogens, mostly composed of archaea, with a time frame of at least ten days. Acidogens and methanogens are also differentiated by the biomass synthesis yield of 0.06-0.12 $\frac{gVSS\ biomass\ produced}{gCOD\ feedstock}$ and 0.02-0.06 $\frac{gVSS\ biomass\ produced}{gCOD\ feedstock}$ [29], respectively. In addition, the phase separation avoids competition, permitting to enhance the biomethane production and the collection of biohydrogen. Biohydrogen is produced also in the traditional mono-stage AD, but it is mainly converted in biomethane by hydrogenotrophic methanogens (Equation 2) [35]. This mechanism is recognized as "interspecies hydrogen transfer" [29].

Through this process, biohydrogen can be produced continuously, unlike other green technologies. For example, green/yellow hydrogen is produced through the electrolysis of water with renewable energy, which is dependent on external influences such as wind or sun. As a drawback, biohydrogen is not produced in pure form, but in combination with biomethane. It therefore requires further post-processing to be used in the chemical industry or refineries. However, like biomethane, biohydrogen mixed correctly can be injected into the gas grid, leading to partial decarbonization, as explained in detail in Paragraph 1.2. and 1.3. [4].

Moreover, returning to the process, the employment of a specific fermentation step during the process triggers the “pre-treatment effect”. The use of specific parameters improves the hydrolysis of recalcitrant compounds, making them more prone to digestion and enhancing the biomethane formation [38].

The process can be integrated into existing plants without the need for disruption, but by adding a second reactor of a smaller size than the current one. In addition, some plants are already equipped with a fermentation step prior to digestion, but are commonly operated with high HRT for the sole purpose of increasing the biomethane yield. In these cases, implementation would be even more accessible by simply changing the operating parameters.

In the literature, several studies were conducted to improve this process and it is already possible to underline the optimal operative parameters for both phases. Unfortunately, the data collected refer limitedly to non-recalcitrant substrates, such as glucose or organic fraction of municipal solid waste (OFMSW). These parameters are better described in Table 2, reporting HRT, OLR, temperature, C/N ratio, and pH value.

Table 2. Optimal parameters for TSAD of easily biodegradable feedstocks

		Fermentation Phase	**Digestion Phase**
HRT	d	0.5 – 3	>15
OLR	$\frac{KgCOD}{m^3 \times d}$	>37	<20
Temperature	-	Mesophilic	Mesophilic/Thermophilic
C/N	-	15-35	50
pH	-	5.5-6.5	>6.5

The process is widely studied in literature either on a lab and pilot scale, reaching encouraging hydrogen results. For example, fermenting OFMSW achieved hydrogen yields of about 66-74 mLH_2/gVS by Micolucci et al. (2018) and Cavinato et al. (2012) [38,39], while values of 42.6 mLH_2/gVS, 99 mLH_2/gVS and 50.2 $mLH_2/gCOD$ were reported using tofu residues [40], rotten fruits and potatoes residues [41], and, cheese whey [42], respectively. The most investigated matrices are glucose, food processing residues, and OFMSW, which are characterized by a high content of fermentable sugar and elevated biodegradability. Nowadays, the application of TSAD on recalcitrant and lignocellulose feedstocks has not been explored at all. For example, regarding agricultural residues (such as straw, manure, pruning, and peels), although are commonly employed in traditional AD plants, there is limited documentation about TSAD [27]. Given the increase in setting new plants established to reach the goals of 35 bcm by 2030, it might be interesting to assess the feasibility of integrating the TSAD process, defining the energy potential, and the sustainability. Consistent with the European Hydrogen Strategy [3,43], it could be employed for the biohydrogen production injectable into the gas grid with biomethane, and the surplus could be sent to co-generation. Against this backdrop, this thesis sought to evaluate the feasibility of agricultural residues as TSAD feedstock, being a matrix of easily obtainability and that due to its vast quantity, is still a cause of disposal issues. The next chapter describes in detail the characteristics of agricultural residues analyzed in this research, reporting their advantages and disadvantages as substrates for the TSAD process. In the figure below, it is schematized the possible integration of the TSAD process in the current gas network economy.

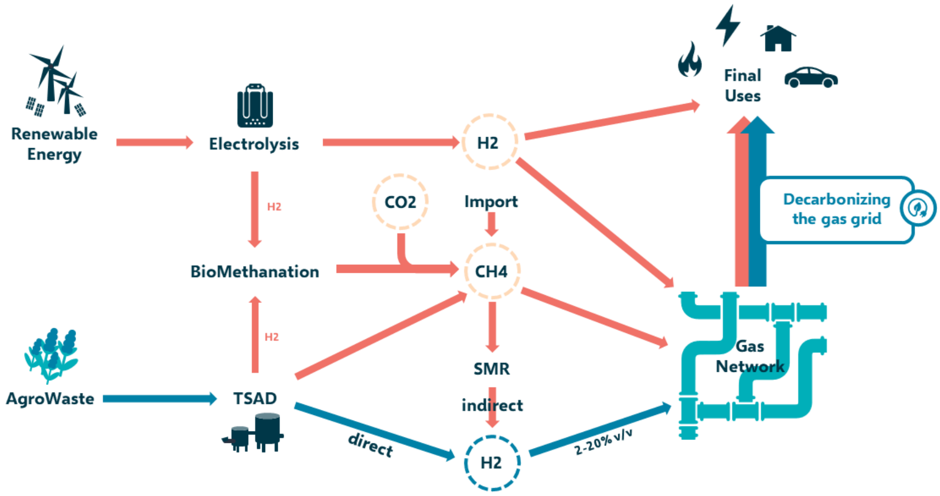

Figure 3. Idealization of TSAD integration into the current gas system

1.5. Agricultural Residues

In Italy, 83,000 agricultural companies (9 million in the EU) are responsible for the production of 18.6 Mt/y dry vegetal residues and 103.2 Mt/y manure and slurries [7]. Inevitable organic waste derived from primary foodstuff generation. For example in EU: wheat (140 Mt/y from 24 Mha), corn (80 Mt/y from 9 Mha), grapes (20 Mt/y from 3 Mha), milk and meat (about 151 Mt/y and 42 Mt/y, from 0.7 livestock unit per ha) [44,45] are responsible for about 123.1 Mt/y of wheat straw, 62.4 Mt/y of corn residues (straw and stalk), 9.1 Mt/y of grape residues (pomace, prunings, and leaves), and, 1.3 Gt/y of livestock manures and slurries [7,46,47].

Agrowaste are mainly characterized by high cellulose, hemicellulose, and lignin, which lead to slow microbial biodegradability. Against this, the vegetal residues have a high C/N ratio, while the animal dejections show a lower ratio due to the important ammonia and urea content. Table 3 below reports the characterizations of common agrowaste, described in terms of macro-nutrients (protein, lipids, and sugars), cellulose, hemicellulose, lignin, starch, nitrogen, phosphorus, and potassium content.

Table 3. Characteristics of common agricultural residues

	Cellulose	**Hemi-cellulose**	**Lignin**	**Proteins**	**Sugars**	**Lipids**	**N**	**P**	**K**
				$\frac{g}{KgTS}$					
Wheat straw	471	270	76	45	16	15	7	1	13
Corn residues	340	303	56	68	12	18	11	2	17
Triticale residue	160	286	58	250	13	92	40	5	0
Grape pomace	148	116	298	148	142	*	19	3	14
Cattle manure	138	186	35	223	*	55	40	17	25
Swine manure	115	95	64	92	*	*	23	8	6

References [47–51]

Unfortunately, the residues are still employed in non-virtuous disposal operations, as incineration and landfill, activities that clearly contrast the aims of the “Landfill Directive” [52] about limiting dumping to 10% by 2035, in particular for items still reusable or valorizable. The most common disposal practice is the deposition as soil conditioners, to contribute to the slow nutrient reintegration for the next cultivation cycles. However, this exercise hides underestimated contraindications, that are aggravated by the wrong performance (such as the employment of unstabilized manure) [7]. Firstly, the biodegradation of agrowaste leads to a direct atmospheric release of GHG, such as carbon dioxide, nitrous oxide, and methane, which contribute directly to the progress of climate change, and waste the opportunity of being exploited again, as in the production of green fuel. Furthermore, ammonia is often released, which contributes to the formation of polluting particles PM 2.5, that are hazardous to human health. The incomplete nutrient adsorption by the soil could be a cause of nitrogen and phosphorus leaching in the aquifers, developing the eutrophication phenomenon that threatens the biodiversity of aquatic species. In addition, traces of chemical compounds such as hormones, antibodies, and pesticides can be found in these residues, and their direct use on the ground contributes to risky environmental diffusion. In particular, the uncontrolled spread of antibodies helps the formation of microbial resistances, a drama that already plagues the hospital sector. Lastly, the employment of unstabilized manure contributes to the pathogen’s diffusion correlated to zoonoses, increasing the risk of “spillover”.

Nevertheless, virtuous practices are already used for agrowaste management. Composting represents the main, with about 3,500 plants in the EU and 320 in Italy

(35 Mt/y and 6.5 Mt/y of compost produced) [53]. Composting is an aerobic process that concerns the conversion of organic fraction into a fertilizer similar to humus (rich in humic and fulvic acid), leading to nutrient recovery and re-employment.

Another widely used practice is the AD, with at least half of the European plants and about 1,100 in Italy involved in the use of this type of feedstock. AD plants are traditionally installed in the same agricultural companies that produce agrowaste (reducing the transport costs and impact), and permit to convert the organic matter into biogas and digestate. In the following table, are reported the biomethane yields obtained from some agricultural residues currently employed in the traditional AD.

Table 4. Agrowaste biomethane yields from AD processes

Feedstock	Biomethane yield		References
Corn residue	$\frac{LCH_4}{KgVS}$	200-240	[5,54]
Triticale residue		145-190	[5,55]
Cattle manure		300-350	[56]
Swine slurry		610-680	[57]
Spent Mushroom Bed		160-310	[58,59]
Wheat straw		160-230	[54,55,60]

The process is doubly interesting for the economy of the farm that integrates it, given the production of digestate. Digestate is a semi-liquid stabilized soil conditioner rich in nutrients as nitrogen and phosphorus, which within certain limits, can be used to fertilize cultivated fields. Some AD plants exposed further virtuosity by employing an “upgrading” phase to remove the CO_2 and H_2S from biogas (usually done by chemical membranes), collecting pure biomethane. Biomethane, contrary to the biogas that is destined for co-generation (electrical and thermal energy production), can be injected into the gas network or used as raw compounds for chemical industries. Currently, agrowaste are the main feedstock used for biogas and biomethane production, but needs some precautions due to its lignocellulosic nature. It is necessary to achieve a suitable hydrolysis, being the limiting step for AD, and in the case of recalcitrant matrices is basically longer (compared for example to the use of sugar or OFMSW as substrates). In addition, an optimized C/N ratio and micronutrient content permit to avoid microbial suffering conditions, and to reach the desired gaseous yields. These details explain the prevalent use of co-digestion in existing plants, acted through the mixing of carbonaceous material (as straw/stalk/prunings) with manure or slurries rich in nitrogen and micro-nutrients. The employment of manure to decrease the C/N ratio exposes the process to ammonia inhibition risk, so it is fundamental to adopt the correct balancing. In the studies of TSAD process, the employment of agrowaste is still under investigation due to the need to optimize the operative parameters both for the digestion and DF phase [7,27,47].

1.6. Aim and objectives of the thesis

Given the information reported in the previous paragraphs, the thesis deals with the TSAD process of agrowaste for biomethane and biohydrogen production, destined for the gas network injection. The research focused on process feasibility and optimisation of biohydrogen production, through the study of different operational parameters and conditions, especially HRTs.

The choice of raw material used in the tests was not accidental, but consistent with the availability of the Veneto region, the location of University of Verona.

The thesis is structured in:

Chapter 2 – Feedstock pre-treatments

The section is dedicated to the importance of the feedstock utilized. It reports an experiment on the efficiency of some pretreatments as methods to improve the fermentation phase.

Chapter 3 – Livestock effluents

The section is dedicated to the employment of livestock dejections as fermentation feedstock, and the possible issues related to high ammonia and methanogens contents. The first part is a preliminary TSAD test on swine slurry. The second is a fermentation study at different HRTs on cattle manure.

Chapter 4 – Vegetal feedstocks

The section is dedicated to the application of vegetal residues, and how co-digestion or alternative strategies could be essential to exceed the limit dictated by nutrient scarcity. The chapter reports two fermentation studies, the first on residues of mushroom cultivation and the second on corn residue and spoiled soybean meal.

Chapter 5 – Economic analysis

The final section is dedicated to the economic evaluation of the real employment of TSAD as a new plant and as a DF-phase-only integration. The evaluation is based on the results collected during the whole study and some literature values.

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2. Feedstock pre-treatments

2.1. Introduction

The feedstock nature importantly affects the AD process, in particular determining the time of the reaction and the bioconversion efficiency. Longer timelines turn out to be inconvenient for the economy of the plant, slowing down the agrowaste degradation and the biofuels production, also increasing the risk of accumulation. Moreover, the costs increase such as for heating and maintaining the reactors. For these reasons, it is essential to optimise the timing of the process. Even because, as reported by Shanmugam et al. (2021) [1], feedstock bioavailability is crucial for biohydrogen production.

As previously reported in Table 3, the agricultural residues are composed of lignocellulose, a rigid three-dimensional polysaccharide structure composed of cellulose, hemicellulose, lignin, pentoses and hexoses, characterised by crystallinity and low solubility. Range of characteristics that make agrowaste recalcitrant. In particular, the lignin compound is rarely biodegraded by bacteria, leader microorganisms of DF.

In literature, is already well-known the use of pre-treatment to improve the bioavailability of organic matter through physical, chemical or biological methods [2,3]. The employment of pre-treatments acts at different layers to make the compound more bioavailable: I. Increases the specific surface, creating multiple points of attack for microorganisms; II. Breaks the lignin-carbohydrate bonds; III. Reduces the crystallinity level; IV. Partially solubilises the lignin links [1].

Physical methods are the most widely used and are mostly based on fragmenting the biomass and reducing its size by high mechanical forces, pressures and temperatures. The steam explosion is based on increasing the porosity and the surface of the feedstock, saturating the biomass with water vapour at high temperatures (170 °C-210 °C) and pressures, and then causing its internal explosion by dropping the pressure to atmospheric value [4]. Hydrodynamic cavitation differs only in the absence of vapour and in the passage of biomass through a small hole, which causes the formation of microbubbles within the fibres. The use of ultrasound allows the formation of cavitation bubbles that cause the internal breakdown of biomass. Microwave pretreatments exploit heat generation to break hydrogen bonds in lignocellulosic matter and to degrade the polysaccharide structures. Bead milling pretreatment uses ceramic, glass, steel or quartz spheres (0.3-0.4 mm) at high speed, able to shear the biomass through mechanical collisions. The pulsed electric field can be used to generate cavities on the cell membrane. Freezing the biomass is often recognised as a pre-treatment, due to the cells' internal breakdown caused by ice crystals formation [1]. However, one of the most common disadvantages of physical pre-treatment is the release of reactive oxygen species (ROS), which can hinder the fermentation process.

Chemical pretreatments are based on the use of acidic (HCl, H_2SO_4), alkaline (KOH, NaOH, $Ca(OH)_2$), oxidants or solvent substances, which can alter the chemical nature of lignocellulose. Despite their high effectiveness, they are costly and potentially environmentally hazardous. Therefore, the use requires recovery at the end of treatment for reuse, resulting in lower costs and avoiding downstream process inhibition [1].

Biological pretreatments are based on the use of enzymes (cellulases, hemi-cellulases, laccases, peroxidases, oxidases), microorganisms (as soft-rot, brown-rot, and white-rot fungi) or microbial consortia [5]. They are highly effective, but in most cases require the use of specific conditions such as pH, temperature, and time. The main limitation is their high cost.

In general, pre-treatments have high costs. Therefore, the implementation must be justified by a major process improvement. Several studies reported interesting results, also for the fermentation phase, able to justify the use of specific pre-treatment on substrates: Kim & Lee (2011) [3] managed to enhance four times the biohydrogen yield from tofu residue through an acid pretreatment, Leaño & Babel (2012) [6] improve bioH_2 yield of 8.8 times with the integration of an alpha-amylase before the tests on cassava wastewater, and, with an ultrasonic-acid combined pretreatment, Elbeshbishy et al. (2011) [7] achieved an improvement of 56.5% in biohydrogen yield from food waste. In this regard, it was decided to test the efficiency of agrowaste' pretreatments for DF.

In the following paragraphs, the methods and the results are better described.

2.2. Materials and Methods

Based on feedstocks' bioavailability, three agricultural residues were chosen for the pre-treatment analysis: corn residue, triticale residue and swine slurry. As representatives of plant and animal derivatives. Following the findings outlined in the next section, no further testing was carried out on other types of agrowaste. The agrowaste and an agricultural digestate, used as mixed inoculum, were recovered from an agricultural company "Cooperativa Agricola Zootecnica La Torre", Isola della Scala (VR), Italy. The substrates and the inoculum were characterized in terms of TS and total Volatile Solids (VS) in accordance with IRSA CNR Standard

Methods [8]. Acid (AC) and Acid-Enzymatic (AE) pre-treatments were evaluated and compared to the Control (CTR). AC was performed by decreasing the pH to 2 adding 5% v/v of H_2SO_4 to the feedstocks in a glass bottle and letting it rest overnight. After that, the pH was adjusted to the neutrality with NaOH. For AE a further step was performed: adding a 1% in VS of Cellulase Enzyme (from *Aspergillus niger*, Sigma-Aldrich), but in that case, only vegetal residues were tested. Swine slurry was excluded from this pretreatment, being a substrate already digestate by the animal's intestinal enzymes.

Feedstocks pretreated were then tested through Biomethanation Potential (BMP) tests [9] facilitating the first step of fermentation, as in TSAD, using a substrate:inoculum ratio of 5 in VS. The ratio used was in fact more unbalanced to the substrate compared to the value of 0.25, commonly employed for this type of test. BMP tests were conducted in triplicate using a 1 L sealed glass bottle, with half operative volume (composed by a 0.3%-0.4% w/w TS), maintained at 38 °C in a heating room for at least 40 days. Daily gas production was quantitatively measured through the water displacement method, and through the data, production curves were built and Specific Gas Productions (SGPs) calculated, as in Equation 5 below. Finally, the results were compared with the CTR.

$$Specific\ Gas\ Production\ \left(\frac{NmL_{gas}}{gVS}\right) = \frac{Average\ \ daily\ gas\ production\ (\frac{NmL_{Gas}}{d})}{Daily\ VS\ input\ (\frac{gVS}{d})} \qquad \text{(Eq.5)}$$

2.3. Results and Discussion

Once feedstocks and the inoculum were collected, the characterisation was performed, and the results are specified in Table 5 below. Swine slurry, differently from the other two substrates, reported a very low solids concentration indicative of a more diluted nutrient concentration. In addition, the lower VS/TS ratio indicates a high amount of non-biodegradable material (about 29% w/w), probably composed of residual salts (as well as in digestate) and stones derived from pig farming.

Table 5. Agricultural Digestate, corn residue, triticale residue and swine slurry characteristics in TS%, VS%, VS/TS%

	TS (% w/w)	VS (% w/w)	VS/TS (% w/w)
Agricultural digestate	5.3±0.1	3.3±0.1	63.0±0.3
Corn Residue	27.8±0.3	23.6±0.1	84.9±1.2
Triticale Residue	28.7±0.3	24.9±0.5	86.8±0.7
Swine Slurry	3.7±0.0	2.6±0.0	71.2±0.1

Following the characterization, BMPs were performed, and the production curves are shown below, in Figure 4. The SGPs were calculated for each feedstock, reporting: for corn residue CTR 100.4 NLgas/KgVS, AC 77.8 NLgas/KgVS, AE 104.3 NLgas/KgVS; for triticale residue CTR 131.7 NLgas/KgVS, AC 40.6

NLgas/KgVS, AE 180.0 NLgas/KgVS, and, for swine slurry CTR 173.6 NLgas/KgVS and AC 76.1 NLgas/KgVS.

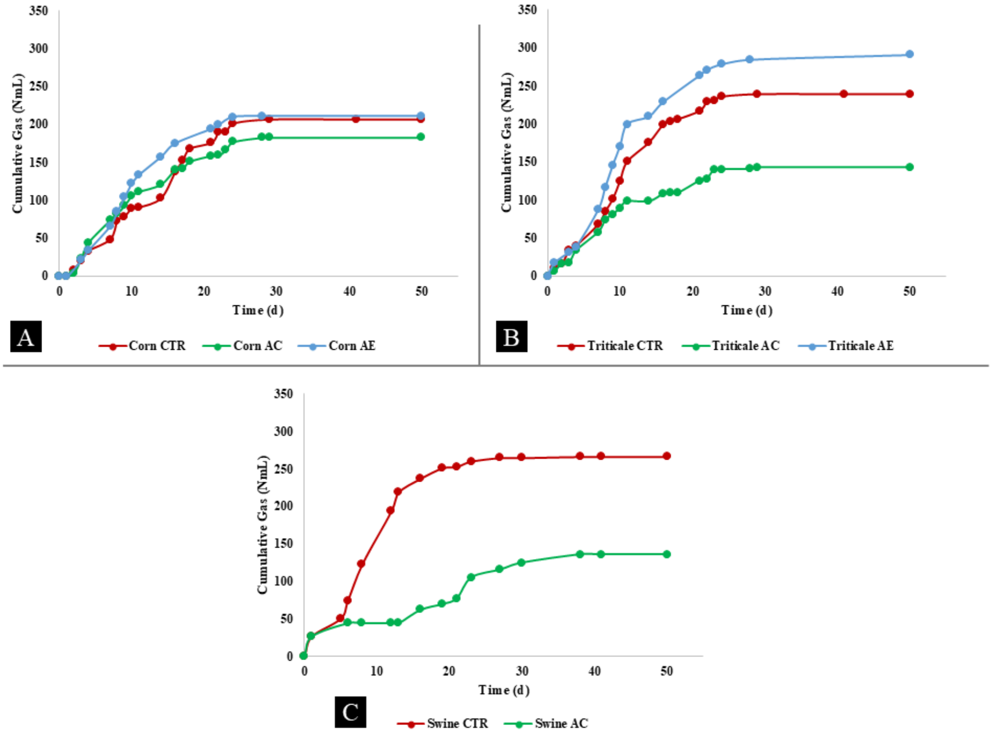

Figure 4. BMPs' production curves (A.corn residue; B.triticale residue; C.swine slurry)

Between the untreated feed tests, swine slurry showed the highest SGP, a result justified by the higher biodegradability given by the non-lignocellulosic nature. In fact, swine are monogastric animals not able to digest fibre, so the farm feedings are mainly based on cereals (wheat, barley, corn) and protein meals.

Contrary to the literature [10–13], the AC pretreatment in all cases reported lower SGPs compared to the untreateds (-23% for corn residue, -69% for triticale residue, -56% for swine slurry), and that results could be due to the formation of inhibitory compounds, such as furfurals, hydroxymethylfurfural, and H_2S, derived from H_2SO_4 utilization.

Whereas the addition of cellulase on plant residues increased the SGPs results, in particular of 4% and 37% for corn and triticale residue, respectively. Likely, the use of the enzyme on triticale residue still managed to facilitate the hydrolysis of the lignocellulosic compounds, and consequently increased gas production; furthermore, counteracting the negative effect of inhibitory compounds. Reaching values comparable to the 1%-33% increase in biomethane production reported by Stanley et al. (2022) [10].

Moreover, as it is visible from Figure 4, AE pretreatment of triticale managed also to accelerate gas production, about 75% of the total was generated in the first 10 days, while the untreated needed 13 days.

In conclusion, it was decided to abandon the use of pretreatment in subsequent tests, given the discouraging results obtained from AC treatment and the high costs involved in the continuous use of an enzyme. This choice, as better discussed in the following chapters, made it possible to evaluate the use of a DF phase as a pre-treatment [14].

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3. Livestock effluents

3.1. Introduction

As previously reported in Chapter 1, annually a large quantity of manures and slurries is produced (1.2 Gt/y [1]), and the incorrect disposal is correlated to a series of problems for health and environment. The AD process is one of the most virtuous strategies used to dispose and revalorize that agrowaste. In particular, the conversion yield is about 300-350 m^3 of biomethane per ton [2] and it is estimated that the entire application in the AD process could produce up to 22 bcm [3]. Upgrading the AD process to the double stage could be an interesting strategy to improve methane yield and collect biohydrogen from using manures, but literature on this subject is scarce. For this reason, TSAD of manure has been a topic of this study. Firstly, evaluating the feasibility of the process on a lab-scale, and later in an attempt to increase its effectiveness.

The first analysis comprehended the utilisation of swine slurry as feedstock for a continuous TSAD test, performed in two sequential CSTRs. While for the second test, the focus was on optimizing the DF phase with semi-continuous reactors, and cattle manure as raw material. Downstream of the process, the biomethanation potential of fermented output was compared with the result of fresh substrate (unfermented), to verify the “pre-treatment effect” of the organic matter [4].

3.2. Materials and Methods

Preliminary TSAD test with swine slurry

Once feedstocks and agricultural digestate were collected (from “Cooperativa Agricola Zootecnica La Torre”, Isola della Scala (VR), Italy), have been characterized in terms of TS, VS, Chemical Oxygen Demand (COD) and Total Kjeldahl Method (TKN) following the IRSA CNR Standard Methods [5]. In particular, solids were analysed by gravimetric method, while COD and TKN by titration. Consequently, it was possible to set the TSAD preliminary test. Swine slurry was chosen as substrate and the digestate as mixed inoculum. The process was carried out for 65 days in two CSTRs of 5 L and 10 L ("automatically-fed digester systems, © Anaero Technology"), respectively, for DF and the methanogenic phase.

Figure 5. Automatically-fed digester systems

In Table 6 below, operative parameters are better detailed. Temperature was maintained controlled at 37 °C by heating jackets. Due to the different characteristics of swine manures used, two analytical periods were distinguished by OLR, the first from the starting day to the 25th, when the second started. No water was added to the process. The aim of the test was not to optimize the process by adjusting the operative parameters, but only to evaluate the lab-scale feasibility using mesophilic temperature and a lower HRT for the first phase compared to the second.

Table 6. Operative parameters and conditions of TSAD preliminary test with swine slurry

	Unit	Dark Fermentation	Methanogenic phase
HRT	d	4.2	28.6
OLR (1st period)	$\frac{gCOD}{(L \times d)}$	7.2	0.5
OLR (2nd period)	$\frac{gCOD}{(L \times d)}$	1.5	0.2
Temperature	°C	37	37
Starting pH	-	5.8	8

Each day, the volume of gases produced from both reactors was registered through the water displacement method. Gaseous samples were collected and quantitatively analyzed by a "Geotech Biogas 5000 analyzer", in terms of CH_4 (% v/v), CO_2 (% v/v), H_2S (ppm), O_2 (% v/v), Residual Gases (% v/v). Hydrogen content (% v/v) was indirectly estimated based on the content of the recorded gases and the usual atmospheric composition (78% nitrogen and 21% oxygen), as in Equation 6 below.

$$H_2\,\% = Residual\ gases\,\% \ - N_2\,\%$$

$$H_2\,\% = Residual\ gases\,\% \ - (\tfrac{78\%\ \times\ O_2\,\%}{21\%}) \qquad \text{(Eq.6)}$$

The analyser reports as "Residual Gases" all the gases that is not able to identify. Considering that the tests were conducted in borderline conditions between acidogenic fermentation and methanogenesis, only nitrogen (N_2) and hydrogen (H_2) were expected to be found in the residual gaseous mixture. Consequently, obtaining the nitrogen content was possible to estimate the hydrogen content. Nitrogen content, derived entirely from the air left in the reactor, was calculated through a proportion between the relation with oxygen in the atmosphere (78% N_2 : 21% O_2) and the effective oxygen found by the analyser.

The gaseous yields as SGP (Equation 5), Specific Methane Production (SMP) (Equation 7), and Specific Hydrogen Production (SHP) (Equation 8) were calculated based on VS (NmL/gVS). Also, liquid samples were collected daily and COD content was verified.

$$Specific\ Methane\ Production\ \left(\frac{NmL_{CH_4}}{gVS}\right) = \frac{Average\ \ daily\ methane\ production\ (\frac{NmL_{CH_4}}{d})}{Daily\ VS\ load\ (\frac{gVS}{d})} \quad \text{(Eq.7)}$$

$$Specific\ Hydrogen\ Production\ \left(\frac{NmL_{H_2}}{gVS}\right) = \frac{Average\ \ daily\ hydrogen\ production\ (\frac{NmL_{H_2}}{d})}{Daily\ VS\ load\ (\frac{gVS}{d})} \quad \text{(Eq.8)}$$

Optimization of DF with different HRTs on cattle manure

As the agricultural digestate, cattle manure was recovered from "Cooperativa Agricola Zootecnica La Torre", and characterized in terms of TS, VS, COD, and TKN, as detailed reported in the results section. 1 L glass "Duran" semi-continuous reactors were employed to optimize the DF with cattle manure, testing three HRTs: 3 d (14.65 gVS/(L*d)), 7 d (6.28 gVS/(L*d)), and 10 d (4.4 gVS/(L*d)). The use of high HRT values, as 7 and 10 days, was established considering the necessity of more time for lignocellulose degradation compared to non-recalcitrant feedstocks (as OFMSW and simple sugars). The use of smaller reactors for the DF optimization was due firstly to the necessity to work with more comfortable sizes, and secondly, to the high solid content of manure substrate, not suitable for the automatically-fed digesters' configuration (prone to clogging problems).

Figure 6. Glass "Duran" semi-continuous reactors setup

Agricultural digestate was used as an acidogens and methanogens inoculum. Daily, reactors were opened to remove the output and insert the fresh substrate, and also, favoring the "Venting" effect [6]. Temperature and pH were controlled with heating jackets at 38 °C and a pHmeter "Mettler Toledo©", adding HCl or NaOH as needed between the range of 5.5 and 6.5. The tests were mixed with an orbital shaker and a TS limit of 5% w/w was imposed to avoid clogging problems. Daily solid-liquid samples were collected and solids, VFAs, COD, and TKN concentrations were monitored. In particular, for the acids, an ionic chromatographer was used, "Thermo Fisher Scientific ICS-1100". The gases produced were collected in gas bags through one-way valves, and, quantitively and qualitatively analyzed by water displacement methods and a “MicroGC Fusion Gas Analyzer (INFICON)”, reporting H_2 (% v/v), CH_4 (% v/v), CO_2 (% v/v), O_2 (% v/v), N_2 (% v/v), H_2S (% v/v). A metagenomic analysis for HRT 3 d was conducted by the laboratory “BMR Genomics s.r.l.” (Padova, Italy), through the identification of the 16s rRNA (bacteria and archaea), with NGS sequencing. The analysis aimed to verify the presence of microbial species more specific to the DF or methanogenic phase. Finally, the biomethanation potentials of the fermented outputs were compared with the results of the fresh substrate. The BMP tests were carried out using 1 L glass sealed bottles with 0.5 L of operative volume (composed by a 2.3% w/w in TS), maintained for 50 days in a heating room at 38 °C, and using an S:I ratio of 0.25 [7]. As the fermentations, the digestate constitutes the inoculum. The tests were done in triplicate, evaluating the daily gas production and the gaseous mixed composition, with the same methods as the fermentations. Once concluded, the gas production curves were built, and SGP (Equation 5) and SMP (Equation 7) were calculated and compared. In the following paragraph, the results are exposed and discussed.

3.3. Results and Discussion

Once collected, feedstocks and agricultural digestate were characterized (Table 7). As expected from the visible appearance, the solids content of swine slurries was more similar to the digestate, compared to cattle manure. While being of a more solid nature, it was preferred to describe cattle manure based on weight (g/KgTS and g/Kg) rather than volume (g/L). As mentioned, two swine slurry samples were employed for the preliminary test, the second has not maintained consistency both in terms of VS and especially COD, decreasing by 5 times. Furthermore, the raw materials of animal derivation exhibit a low C/N ratio, being dejections rich in amino acids, urea, and ammonia. Following the literature, ammonia accumulation can be an inhibitor of the fermentation and digestion phase [8]. For this reason, commonly in the AD process manures and slurries are mixed (in co-digestion) with carbonaceous materials (such as straw, corn cobs, and prunings), to work with a more suitable C/N ratio.

Table 7. Characterization of agricultural digestate, swine slurry, and cattle manure (TS, VS, COD, TKN)

	TS	**VS**	**VS/TS**	**COD**	**TKN**
	% w/w	% w/w	% w/w	gCOD/L	gN/L
Agricultural digestate - Preliminary test	5.3±0.1	3.3±0.1	63.0±0.3	19.4±0.4	7.9±0.1
Agricultural digestate – Fermentation and BMP	6.3±0.0	3.7±0.0	58.97±0.3	26.5±0.3	12.0±1.7
Swine Slurry 1st period	3.7±0.0	2.6±0.0	71.2±0.1	30±2.4	3.8±0.3
Swine Slurry 2nd period	1.9±0.1	1.2±0.1	62.5±1.5	6.3±3.4	2.2±0.4
	% w/w	% w/w	% w/w	gCOD/KgTS	gN/Kg
Cattle Manure	17.4±1.4	15.3±1.2	87.9±0.2	763.5±78.7	14.6±0.3

Preliminary TSAD test with swine slurry

The TSAD process was monitored daily in terms of VS and COD content, and as visible from Figure 7, the difference between the two slurries affects both phases. The diminution in COD determined also a decrease in OLR, which could slow down the fermentation and decrease the gases yield.

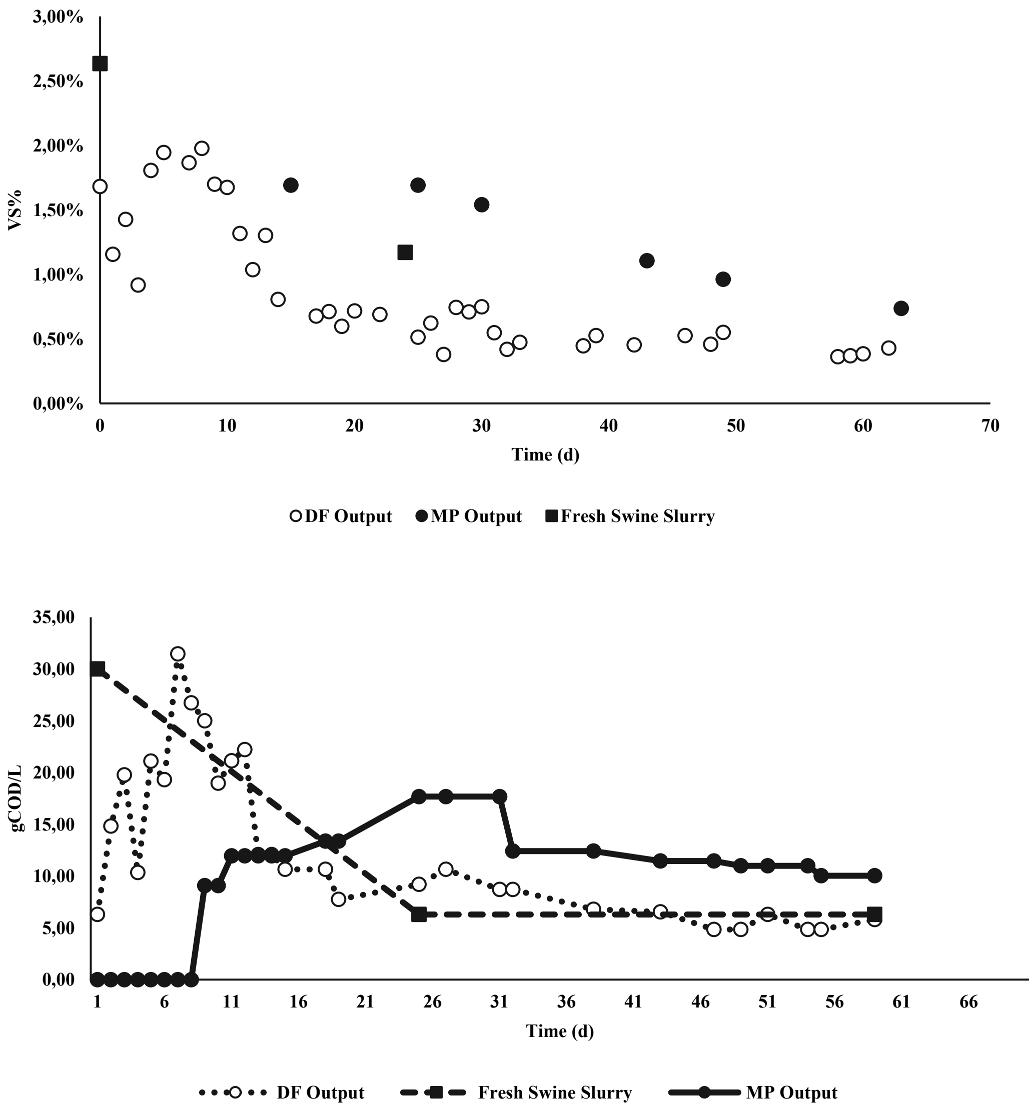

Figure 7. Trends of VS and COD during the test

The gas productions were registered and reported in terms of SGP (Lgas/KgVS) in Figure 8. The first phase reached twice the stability in gaseous production: first time at about the 10th day, with an average of 42.8±5.3 NLgas/KgVS, and then on the 26th day, when it slightly increased to 46.4±3.8 NLgas/KgVS. So, for DF the switch of slurries did not affect the gas yield. The SGP of the second phase needed more time to achieve stability, about an HRT and a half (41 days) and reached 155.3±0.5 NLgas/KgVS. In detail, before day 28th the reactor had yet to be completely filled to its operational volume, so this was probably the reason for the inconsistent gas production.

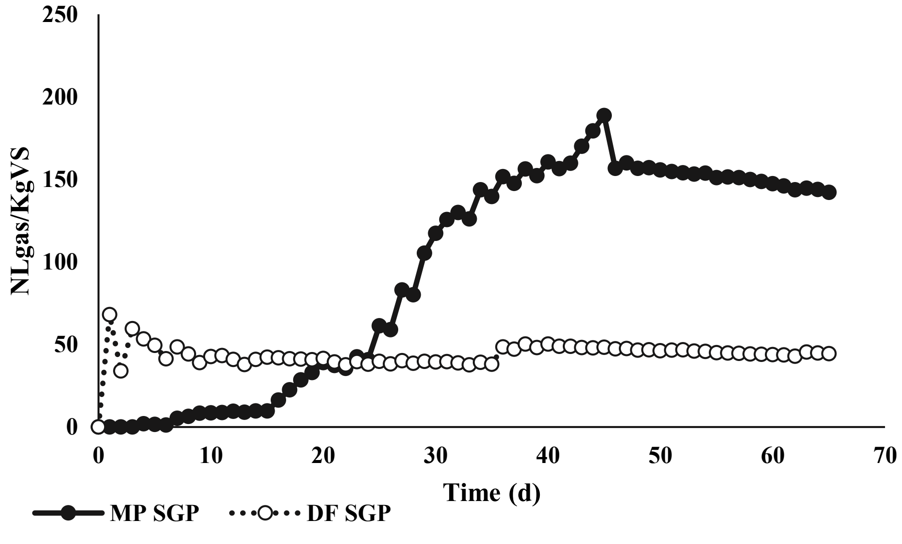

Figure 8. SGP trends for DF and MP phases.

After that, the gases were analyzed to evaluate the specific compositions. It was estimated that the average DF gas was composed of 19% v/v of biomethane (SMP 8.5 $NLCH_4$/KgVS), 18% v/v of carbon dioxide and 63% v/v of biohydrogen (SHP 28.2 NLH_2/KgVS), while for the second phase 53% v/v methane (SMP 82.3

NLCH_4/KgVS) and 47% v/v carbon dioxide were identified. Assuming a total removal of CO_2, the whole process was able to generate 118.9 NL/KgVS of biohythane, composed of 76.3% v/v of CH_4 and 23.7% v/v H_2. A recent study by Chen et al. (2021) [8], reported a TSAD process with rice straw and pig manure, working with thermophilic DF and mesophilic MP, that achieved a SHP of 16.7 LH_2/KgVS and a SMP of 197.7 LCH_4/KgVS. Values comparable to those obtained from that analysis. In particular, the methane yield appears to be higher than the obtained values, probably due to a better balancing of the C/N ratio (due to straw as co-feedstock) and a more efficient hydrolytic and acidogenic step, obtained at thermophilic temperatures. In addition, the estimated hydrogen yield can be recognized as reliable, seeing the small discrepancy with those of the compared study.

Optimization of DF with different HRTs on cattle manure

For each fermentation test was registered the daily gas production and was reported in Figure 9 in terms of H_2 and CH_4. Also, pH trends were reported, in order to highlight the maintenance of optimal value between 5.5-6.5. In all the trials no biohydrogen production was identified, but contrary biomethane started generating after about one HRT. In detail, the SMP measured were decreased following the HRT values: 3.35 NmLCH_4/gVS (HRT 10 d), 1.42 NmLCH_4/gVS (HRT 7 d), 0.61 NmLCH_4/gVS (HRT 3 d). Four reasons could be the cause for hydrogen absence and methane appearance: I. The high recalcitrance of manure is not suitable for fermentation; II. The high charge in methanogens of the substrate leads to an important competition with the acidogens; III. The methanogens' flow probably helps the growth of hydrogen-consuming species, which rapidly convert all the H_2 into CH_4 [9]; IV. The ammonia inhibition. Concerning the ammonia inhibition risk, in the three tests, no one has shown an ammonia concentration above the inhibition threshold of 3.0-8.0 gN/L [8,10]. So, ammonia inhibition was rejected as a possible cause.

Similar to these results, Hangri et al. (2024) [11] reported a negligible hydrogen yield of 0.76 mLH_2/gVS from dairy manure, in particular using a pre-treated inoculum (105 °C for 1.5 h) to avoid methanogens. However, with the application of co-digestion with cheese whey (20% v/v), managed to achieve 10.38 mLH_2/gVS. For these reasons, co-digestion was adopted for a fourth test, in an attempt to improve the process. Corn residue 80% in VS was mixed with cattle manure, and the gaseous results are shown in Figure 9. Corn residue characterization is reported in Table 8. The co-digestion helped the process to generate biohydrogen for one HRT (reaching up to 7.12% v/v on the third day), and then the methanogens prevailed, switching to methane production. Therefore, it was not possible to maintain stable biohydrogen production during the process. Furthermore, it is not viable to compare this trend with Hangri et al. (2024) [11] results, due to their limited time of analysis of 5 days in batch conditions.

Table 8. Corn Residue characterization (TS, VS, COD, TKN)

	TS	**VS**	**VS/TS**	**COD**	**TKN**
	% w/w	% w/w	% w/w	gCOD/KgTS	gN/Kg
Corn Residue	33.7±4.6	31.7±4.7	94.1±1.6	772.1±125.9	1.9±0.0

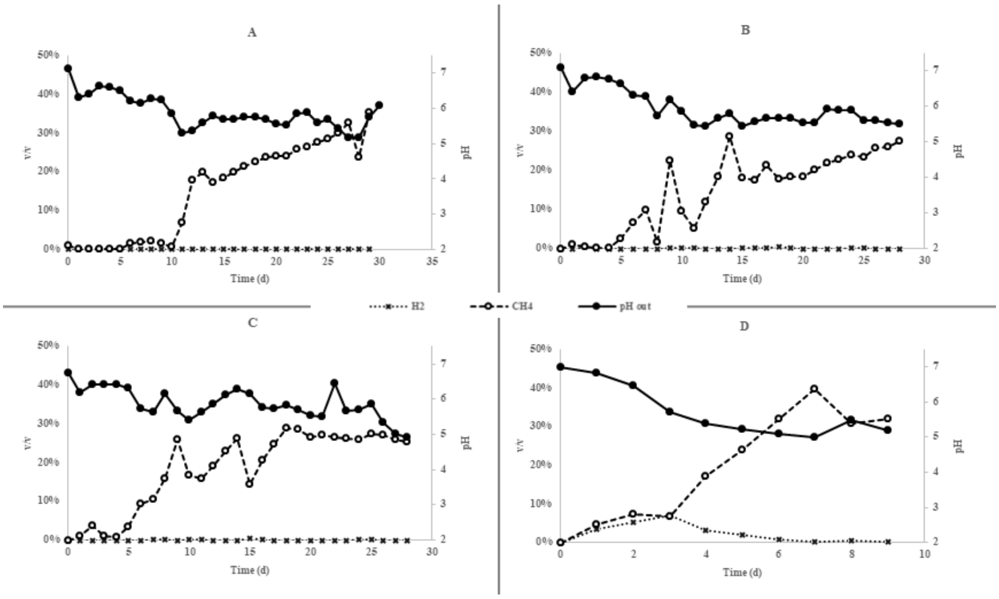

Figure 9. Hydrogen, methane, and, pH trends during cattle manure fermentation (A. HRT 10 d; B. HRT 7 d; C. HRT 3 d; D. HRT 3 d co-digestion).

However, the occurrence of fermentation cannot be excluded given the detection of discrete VFA concentrations, i.e., 5.11 gCOD/L for HRT 10 d, 5.28 gCOD/L for HRT 7 d, and, 4.44 gCOD/L for HRT 3 d. The main acids identified were acetic, butyric, and propionic, as reported in Figure 10. Acetic and butyric are typical products of sugars' DF, as reported in the literature [12,13], while propionic is usually formed from the lipids and proteins conversion [14,15].

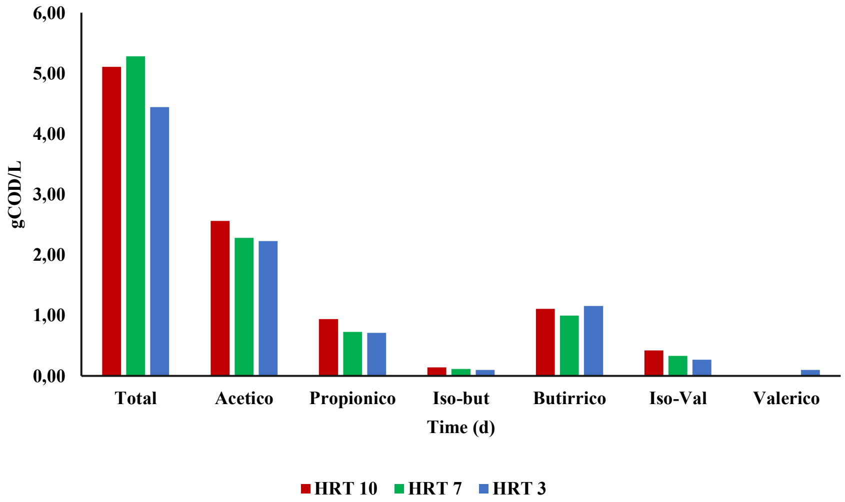

Figure 10. VFAs' concentration of cattle manure fermented outputs

The metagenomic analysis on HRT 3 d output confirmed what was hypothesized, methanogens presence in the reactor. In particular, *Methanobrevibacter* (belonging to the *Methanobacteriaceae* family) was 21% of the total relative abundance, and it is known for its methane generation capacity [16–19]. The finding of methanogens at the lower HRT tested confirms the possibility of finding them also at HRT 7 d and 10 d, i.e., better environments for low growth and low replication kinetics. Although no biohydrogen was detected, the metagenomic analysis found the presence of *Romboutsia* (14.44%) and *Paeniclostridium* (13.57%) (belonging to the *Peptostreptococcaceae* family), bacteria often implicated in the production of H_2 in AD processes involving acid pretreatment [20–22]. Similarly, the 5.29% of *Turicibacter*, an *Erysipelotrichales* bacterium capable of producing biohydrogen and small VFAs from the oxidation of simple sugars, has also been found [23]. Lastly, given the presence of *Prevotella* (4.65%), the absence of hydrogen may be input not only to its conversion to methane, but also to the diversion of its metabolic pathway for the propionate production from polysaccharides and proteins [24].

Although the fermentation test where negative for biohydrogen production, the outputs reported positive results from biomethanation potential. The values exceed the control (fresh substrate 611.01 NmLCH_4/gVS), reaching a methane enhancement of about +18.7% for HRT 10 d (725.48 NmLCH_4/gVS), +11.7% % for HRT 7 d (682.63 NmLCH_4/gVS) and 4.1% for HRT 3 d (635.95 NmLCH_4/gVS). The cumulative methane productions are represented in Figure 11, below. As reported from the results, effectively, the use of a specific fermentation stage upstream of digestion acts as a pretreatment and increases methane yields.

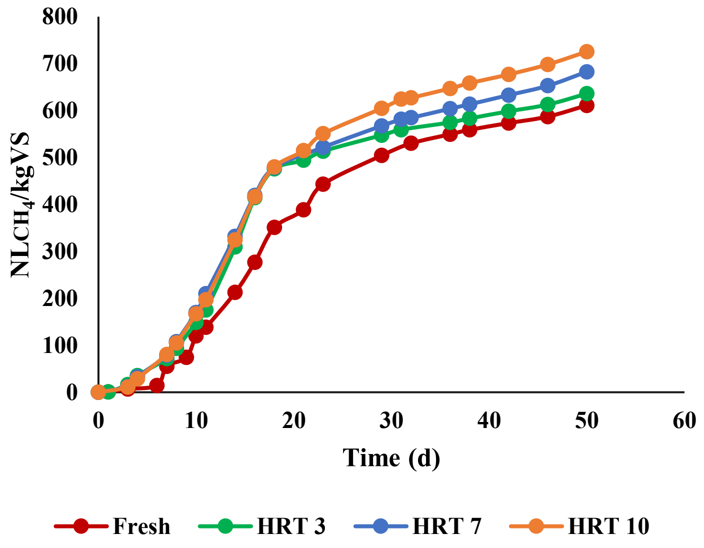

Figure 11. Increase in SMP as the BMP tests progress.

In conclusion, the employment of cattle manure in a TSAD process is not a good choice for biohydrogen production. The inner methanogens content competes with acidogens and converts their generated products. The application of a co-substrate is to be considered necessary to achieve biohydrogen generation, furthermore, other improvements are needed to reach a stable production. Nevertheless, it is technologically important to consider the application of the fermentation step as a real pretreatment to improve biomethane generation, providing a higher organic matter bioavailability and partial bioconversion in VFAs. The application of TSAD could improve the manure's methane yield by up to 19%.

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4. Vegetal feedstocks

4.1. Introduction

As previously mentioned, a large quantity of vegetal agrowaste is produced. To get an idea of their amount, in the EU it is estimated a generation of 123 Mt/y of wheat straw (the most common residue), 60 Mt/y corn residue, and 9.1 Mt/y agrowaste from grapes [1]. As regards Italy, the total dry vegetal scrap is about 18.6 Mt/y, where 77% w/w and 12 % w/w are occupied by straw and prunings, respectively [2]. The potential employment of all these plant residues in AD is estimated to produce 30 bcm of biomethane and biogas [3]. This type of feedstock is characterized by a high content of lignin, hemicellulose, and cellulose, which make it a recalcitrant substrate for microbial biodegradation. As previously reported in Table 3. Due to the lignocellulose characteristic, the employment of plant residues is a challenge and often needs high HRT to be digested in the AD process.

The integration of a specific DF reactor could be a solution to enhance the hydrolysis, the limitation step of the process, and thus improve the biomethane production from lignocellulosic residues. In addition, once optimized could also be used to produce biohydrogen. For these reasons, some tests were performed to evaluate which are the best HRTs for the first step, and if it is possible to improve the gaseous yields.

Spent Mushroom Bed (SMB), corn residue, and spoiled soybean meal were adopted as feedstock for the fermentation tests. Regarding SMB, it is made from exhausted balls of wheat straw and equine manure, previously employed as terrain for mushroom cultivation. Several HRTs were applied to optimize biohydrogen production. After that, the integration of co-fermentation and a recirculation system were investigated in an attempt to maintain the stability of the process. After concluding the fermentation tests, the biomethanation potential of SMB and corn residue was compared with their fermented outputs to verify possible “pre-treatment phenomena”. In conclusion, a kinetic model was applied to SMB tests’ results to evaluate how the OLR affects the VS biodegradation, and a metagenomic analysis was performed on corn residue and soybean meal outputs, to verify the acidogens presence.

4.2. Materials and Methods

The feedstocks (SMB, corn residue, and spoiled soybean meal) and the inoculum were taken from the agricultural company of “La Torre” (Isola della Scala (VR), Italy), and characterized in terms of TS, VS, COD, TKN, and TAN. Given that, the semi-continuous fermentation tests were set up. In particular, several HRT values were studied to determine the best for biohydrogen production from different feedstocks. Seven HRTs (and related OLR) were examined for SMB as substrate, i.e., 1.5 d (29.74 gVS/(L*d)), 4 d (11.15 gVS/(L*d)), 6 d (7.37 gVS/(L*d)), 7 d (6.31 gVS/(L*d)), 10 d (4.46 gVS/(L*d)), 12 d (3.72 gVS/(L*d)), and 18 d (2.48 gVS/(L*d)), while, corn residue fermentation was evaluated with HRT 3 d (15.68 gVS/(L*d)), 5 d (9.41 gVS/(L*d)), and 7 d (6.72 gVS/(L*d)). Glass “Duran” semi-continuous reactors of 1 L were used for all the feedstock’ fermentations. SMB tests were carried out using 0.6 L of working volume, maintaining the reactors at 38 °C in a thermostatic bath, and controlling daily the pH value between 5.5-6.5 with hydrochloric acid and sodium hydroxide. The mixing was granted by a Rushton propeller at 90 rpm, and a TS limit of 5% w/w was imposed to avoid clogging problems. The reactors were opened daily to recover the output and to add new substrate, which was composed of SMB and water. This operation guaranteed the "venting effect" [4]. Each day, gaseous and liquid output were collected for further analysis. In particular, SGP (Equation 5), SMP (Equation 7), SHP (Equation 8) and Gas Producing Rate (GPR) (Equation 9) were calculated from gaseous samples, while VFA yield (Equation 10) and Acids Production Rate (APR) (Equation 11) from liquid samples.

$$Gas\ Producing\ Rate\ \left(\frac{NL_{Gas}}{(L \times d)}\right) = \frac{Average\ \ daily\ Gas\ production\ (\frac{NmL_{Gas}}{d})}{Reactor\ Volume\ (L)} \qquad \text{(Eq.9)}$$

$$VFA\ yield\ \left(\frac{gCOD_{VFA}}{gVS}\right) = \frac{Average\ \ VFA\ concentration\ \left(\frac{gCOD_{VFA}}{L}\right) \times Reactor\ Inflow\ (\frac{L}{d})}{Daily\ VS\ input\ (\frac{gVS}{d})} \qquad \text{(Eq.10)}$$

$$Acids\ Production\ Rate\ \left(\frac{gCOD_{VFA}}{(L \times d)}\right) = \frac{Average\ \ VFA\ concentration\ \left(\frac{gCOD_{VFA}}{L}\right)}{HRT\ (d)} \qquad \text{(Eq.11)}$$

Figure 12. SMB fermentation test setup

For fermentation tests with corn residue and spoiled soybean meal, some modifications were adopted: an operative volume of 1 L (limiting the headspace as much as possible), an orbital shaker for the mixing, a non-return valve was added for gas collection (reducing the risk of hydrogen consumption). Also, the biofilm grown on the headspace walls was removed daily. About spoiled soybean meal was used as 50% in VS co-substrate of corn residue to improve the HRT 3 d test (as a source of nitrogen and microelements). Furthermore, a fermentation test of only soybean residue with HRT 3 d was evaluated to compare the co-fermentation results. Moreover, following the results of corn residue mono-fermentation HRT 3 d reported in Paragraph 4.3, it was chosen to test a recirculation system of a methanogenic reactor output. This addition was done as a buffer system, as reported by Micolucci et al. (2014) [5]. The recirculation ratio utilized was 0.86, calculated following Equation 12. Figure 13 below shows a scheme of the process. Only the supernatant, derived by centrifugation at 3600 rpm for 20 minutes of digestion outputs, was used as a recirculate. The separation was performed to reduce the methanogen content and avoid competition phenomena in the DF reactor. The solid-liquid separation system leads to the decoupling of the HRT value from the solid retention time (SRT) value [6]. In detail, the fermentation reactor operated at SRT 3.0 d, and the output was used as input for a 5 L semi-continuous methanogenic reactor with SRT 15.2 d.

$$Recirculation\ Ratio = \frac{Qr}{Qa} = \frac{Recirculation\ flow\ rate\ (\frac{mL}{d})}{Input\ flow\ rate\ (\frac{mL}{d})} \qquad \text{(Eq.12)}$$

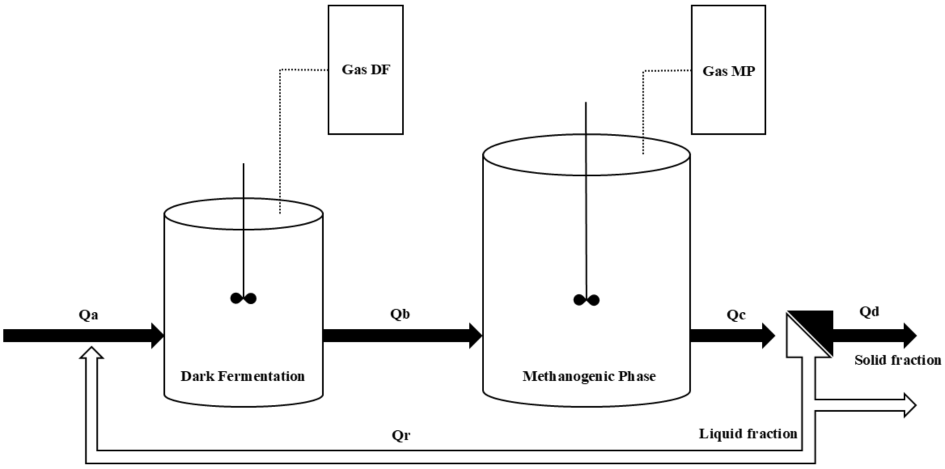

Figure 13. Scheme of HRT 3 d corn residue fermentation with the integration of digestate recirculation.

Liquid outputs of each test were then analyzed in terms of TS, VS, COD, TKN, TAN, and VFAs content, to evaluate the achievement and maintenance of steady state. TAN was also useful to identify any ammonia inhibition phenomena [7,8]. VFAs' content was essential to verify the proper progress of fermentation and also to calculate the conversion efficiency. The analysis was done following the IRSA CNR Standard Methods [9], while the acids content was determined through an ionic chromatograph “Thermo Fisher Scientific ICS-1100”. Gaseous outputs were quantified through the water displacement method. For SMB tests, gas content was estimated through the “Geotech Biogas 5000 analyzer”, as reported in Chapter 3, while for the other trials, using gas chromatography “MicroGC Fusion Gas Analyzer (INFICON)”, with Ar and He as the carriers.

Once SMB fermentation tests were concluded and the data collected, a modified Stover-Kincannon model was constructed. A model usually employed for continuous or semi-continuous anaerobic digestion processes, to predict the rate of degradation of organic matter (VS) in relation to the OLR employed. The construction of this model is an excellent tool from the perspective of a scale-up of the process. As reported by de la Lama et al. (2017) and Nasr et al. (2015) [10,11], this model can be applied not only for the entire anaerobic digestion process, but also for the fermentation phase, as in this case.

The model is built plotting on a Cartesian plane the *HRT* as abscissae and $\frac{HRT}{(So-Se)}$ as ordinates, obtaining the resulting line describable with the Equation 13, reflecting the format $y = mx + q$. In particular, the Equation 13 reports the substrate VS content, as before (*So)* and after fermentation *(Se)*, as gVS/L.

$$\frac{HRT}{(So-Se)} = \frac{Kb}{(Rmax \times So)} \times HRT + \frac{1}{Rmax} \qquad \text{(Eq.13)}$$

Once the line and equation are obtained, it is possible to calculate the kinetic parameters *Kb* and *Rmax* from the slope and the intercept of the trend line. Those values are employable in Equation 14 to evaluate any *Se* resulting in dependence of any *HRT* and *So* applied.

$$Se = So - \frac{Rmax \times So}{Kb+(\frac{So}{HRT})} \qquad \text{(Eq.14)}$$

For fermented outputs of HRT 3 d corn residue, corn residue in co-fermentation with soybean residue, soybean residue, and corn residue integrated with recirculation systems, were performed a metagenomic analysis by “BMR Genomics s.r.l.” (Padova, Italy) and the results were useful to verify acidogens presence and to certify the absence of methanogens.

Finally, the outputs of corn residue fermentation HRT 3 d, HRT 5 d, and HRT 7 d were employed in BMP tests, and the results were compared with unfermented (fresh) corn residue biomethanation potential. The aim was to evaluate whether DF could be recognized as a true pretreatment, able to improve biomethane production during the methanogenic stage. A specific step for hydrolysis is often attributed to a better conversion of macro compounds into more bioavailable ones [12]. In particular, the BMP tests were performed in triplicate in a 1 L sealed glass bottle, with half of the working volume (composed of a 2.3% w/w in TS), and maintained at 38 °C in a heated chamber for at least 50 days. Agricultural digestate was used as inoculum and rationed at a 1:0.25 ratio in VS with the substrate. Daily, the gas production was measured through the water displacement method, and the biomethane content was evaluated through a “Geotech Biogas 5000 analyzer”.

4.3. Results and Discussion

Before starting the fermentation tests, the feedstocks and the agricultural digestate (used as inoculum) were characterized, and the results are better detailed in Table 9. The three feedstocks analyzed were distinguished by the C/N ratio, an essential parameter for the growth of microorganisms. A feedstock with a high C/N ratio, as corn residue, exposes the microbial culture to a nitrogen deficiency, which could lead to a state of distress and block their growth and replication [13–15]. Contrary, a low C/N ratio, as in spoiled soybean meal, reveals a strong balance and ensures nitrogen abundance, but at the same time, it can be a sign of a high ammonia content, which may pose the risk of metabolic inhibition [8]. However, from the reported TAN value of 13 mgN/L in co-fermentation and 26 mgN/L in mono-fermentation, the soybean tests were outside the risk of inhibition, that is about 3.0-8.0 gN/L according to the literature [8,16]. As regards SMB, it reported a 9.1 C/N ratio, which is closer to the optimal for the DF process (about 35.0) [17], which should provide the right amount of nitrogen for acidogens and not exhibit inhibition phenomena. Concerning the VS/TS ratio, it allows to recognize the biodegradable and the inert parts, so as to highlight the fraction that can be attacked by the microbial community. In particular, SMB has the highest inert content of 10.6% w/w, followed by 7.1% w/w of soybean, and finally 5.9% w/w of corn residue.

Table 9. Agricultural digestate, spent mushroom bed, and corn residue characterization (TS, VS, COD, TKN)

	TS	**VS**	**VS/TS**	**COD**	**TKN**	**TAN**	**C/N**
	% w/w	% w/w	% w/w	gCOD/KgTS	gN/Kg	gN/Kg	-
Agricultural digestate	4.1±0.3	2.7±0.0	65.9±4.3	756.1±9.6	12.0±1.7	8.7±0.2	2.5
SMB	29.2±2.4	26.1±2.4	89.4±1.1	866.6±12.7	17.6±1.4	*	9.1
Corn Residue	33.7±4.6	31.7±4.7	94.1±1.6	772.1±125.9	1.9±0.0	0.2±0.0	137.7
Spoiled Soybean meal	88.0±0.2	81.7±0.3	92.9±0.1	912.2±22.2	273.5±0.0	0.4±0.0	2.9

Once the characterization was performed, due to different setups and analyses, the experimentation was divided into two parts. Specifically, dedicated to testing I. SMB and II. Corn residue and spoiled soybean meal.

Spent mushroom bed

The fermentation tests were performed by studying the application of seven different HRT values. And in all the cases, at about one and a half HRT, the process reached the steady state. As can be visible in Figure 14, reporting the pH and gaseous yield trends (NmLGas/gVS). The test HRT 1.5 d was not reported in Figure 14, due to the difficulty of recognizing the steady state from a graphical representation of 5 points.

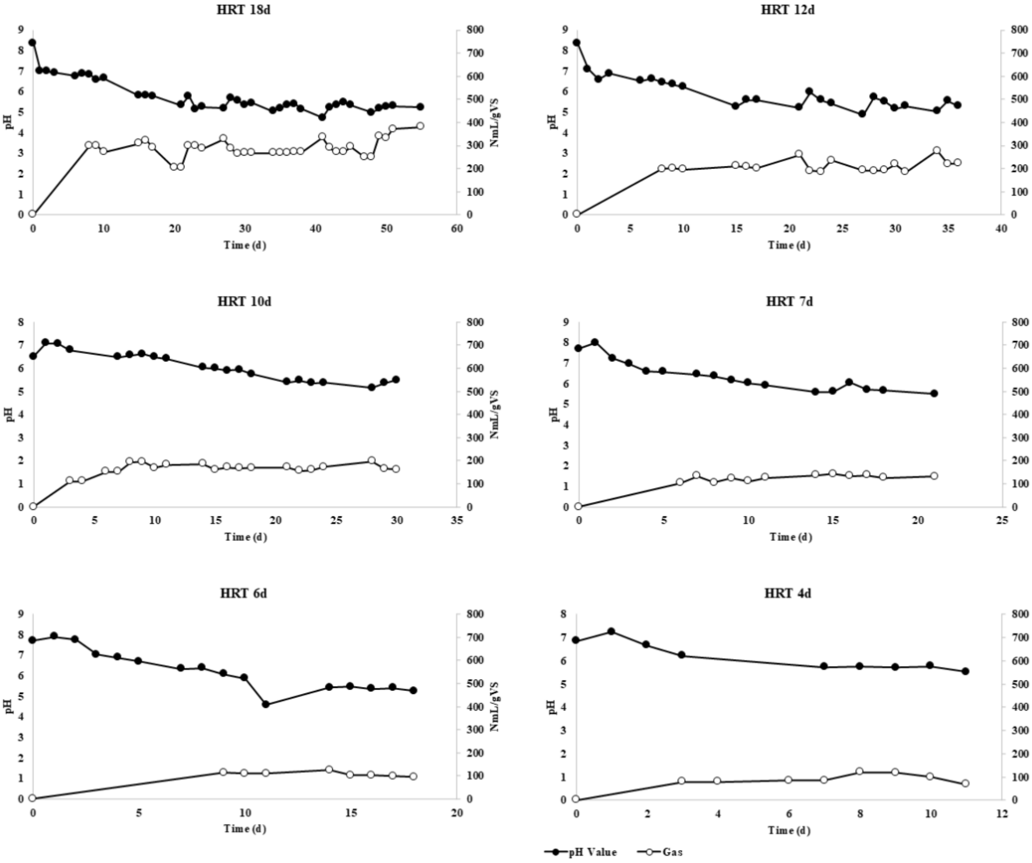

Figure 14. Daily gas production and pH trend for fermentation tests 18 d, 12 d, 10 d, 7 d 6 d, and, 4 d..

Regarding gas production, once the tests were concluded, the average GPR (NLgas/(L*d)) and SGP (NLgas/KgVS) were calculated from the results registered during the steady states. In particular, in Table 10 are reported as total and specific gas fractions (H_2, CH_4 and CO_2). Biohydrogen production being the main target of these tests, HRT 6 d resulted the best, reporting a GPR of 0.41 $NLH_2/(L*d)$. As expected, given the high lignocellulosic content of the straw, longer HRTs were needed to optimize the process, compared with values of about 3 d reported in the literature for other types of more biodegradable substrates. Although HRT 10 d, 12 d, and 18 d reported interesting H_2 yields at about 55 $NLH_2/KgVS$, they cannot be considered as good fermentation due to biomethane production. The biomethane generation is a signal indicating the inevitable transition to a methanogenesis process over longer process times.

Table 10. Gaseous Production Rate and Yields from SMB fermentation tests

HRT	**GPR**		**Yield**			
	Total	H_2	Total	CH_4	CO_2	H_2
d	NLgas/(L*d)	$NLH_2/(L*d)$	NLgas/KgVS	$NLCH_4/KgVS$	$NLCO_2/KgVS$	$NLH_2/KgVS$
1.5	0.85	0.33	23.90	0.00	3.28	9.04
4	0.69	0.37	51.68	0.00	15.06	28.04
6	0.74	0.41	82.62	0.00	22.72	45.88
7	0.61	0.34	80.68	0.00	20.81	43.95
10	0.52	0.29	96.49	0.78	26.53	54.09
12	0.49	0.25	109.92	2.53	39.46	54.97
18	0.36	0.17	122.58	17.17	34.09	57.82

After achieving the steady state, liquid samples were recovered for each test, and the VFAs content was evaluated. Table 11 and Figure 15 report in detail the VFAs profile, APR, and yield from the trials. HRT 4 d showed the highest APR with 0.76±0.1 gCODvfa/(L*d), while the range between 6 d and 10 d optimized the VFAs yield, about 80 gCODvfa/KgVS. At HRT 1.5 d, the VFAs produced are few, while over HRT 10 d, the VFAs generated are simultaneously consumed by methanogens. Different from what was expected, the predominant acids generated were not only acetic and butyric, typical of acidogenic fermentation, but also propionic. From the literature [18,19], propionic generation is usually correlated with lipid and protein conversion, in this case derived from the manure fraction.

Table 11. VFAs profile of SMB fermentation trails (From C2 to C5)

HRT	**Acetic**	**Propionic**	**Iso-Butyric**	**Butyric**	**Iso-Valeric**	**Valeric**
d	gCODvfa/L					
1.5	0.33	0.28	0.00	0.44	0.00	0.00
4.0	1.08	1.02	0.04	0.72	0.15	0.11
6.0	1.30	1.08	0.00	0.93	0.21	0.13
7.0	1.41	1.07	0.06	0.85	0.22	0.07
10.0	1.65	1.01	0.05	0.71	0.23	0.10
12.0	1.19	0.61	0.00	0.90	0.00	0.09
18.0	1.45	0.54	0.00	0.85	0.00	0.00

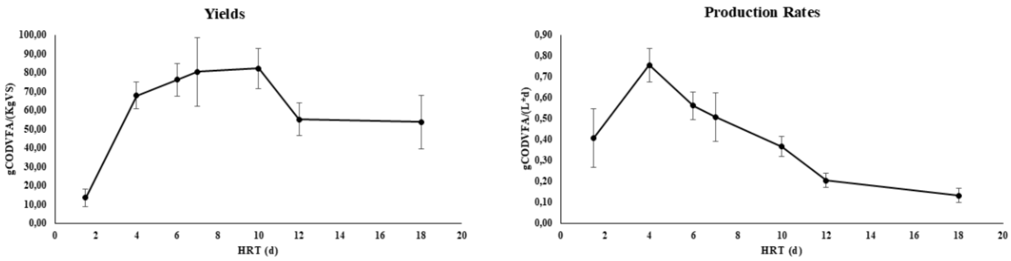

Figure 15. VFAs Production Rate and yield of each HRT fermentation test

The collection of fermentation data at different HRTs permitted the application of a modified Stover-Kincannon model. As reported from the plot of Figure 16 below, the slope ($\frac{Kb}{(Rmax \times So)}$) and the intercept ($\frac{1}{Rmax}$) resulting from the trendline were respectively 0.0413 and 0.0479. With the application of these values, the kinetic parameters of *Rmax* 20.88 gVS/(L*d) and *Kb* 38.46 gVS/(L*d) were calculated. Consequently, *Rmax* and *Kb* application to Equation 14 enables the determination of the theoretical substrate removal at the imposed OLR. This information is useful for a possible construction of a full-scale plant to reduce the quantities of agricultural residues and produce hydrogen, methane, and carbon dioxide [20].

Also, the model reliability was confirmed through the test of the HRTs studied. In particular, reporting an average deviation of 8%, a value lower than de la Lama et al. (2017) [11] threshold of kinetic model functioning, which corresponds to 15%.

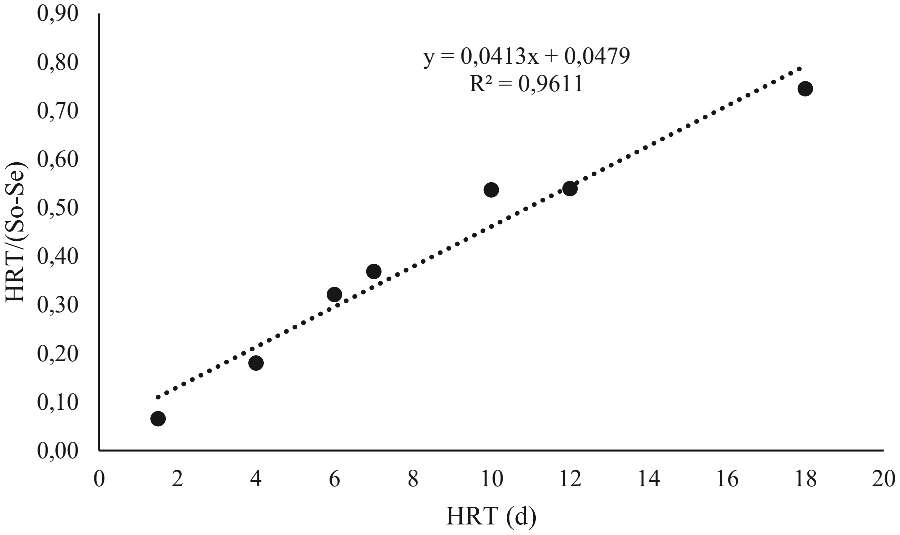

Figure 16. Trendline of the modified Stover-Kincannon model resulting from SMB fermentation data

The fermented outputs were collected and tested in BMP to evaluate primarily if the biomethanation potential could be enhanced from a specific DF phase compared to a fresh SMB, and secondly, which HRT is the most suitable for this phenomenon. The results, reported in Table 12 and Figure 17, highlighted an improvement in biomethane yield compared to the unfermented SMB (i.e., HRT 0 d). The employment of a specific DF phase really acts as a pretreatment for the SMB, permitting a better hydrolysis of the organic matter and its conversion into biomethane. In detail, the highest enhancement was obtained from fermentate HRT 6 d, which increased the biomethane yield by 27% than the fresh SMB.

Table 12. The gaseous potential of the fermented outputs and fresh SMB (HRT 0 d)

HRT	**Total**	**CH_4**
d	NL/KgVS	$NLCH_4/KgVS$
0.0	223.01	154.32
4.0	268.72	180.32
6.0	269.68	196.06
7.0	258.18	173.75
10.0	229.90	148.81
12.0	247.13	115.27

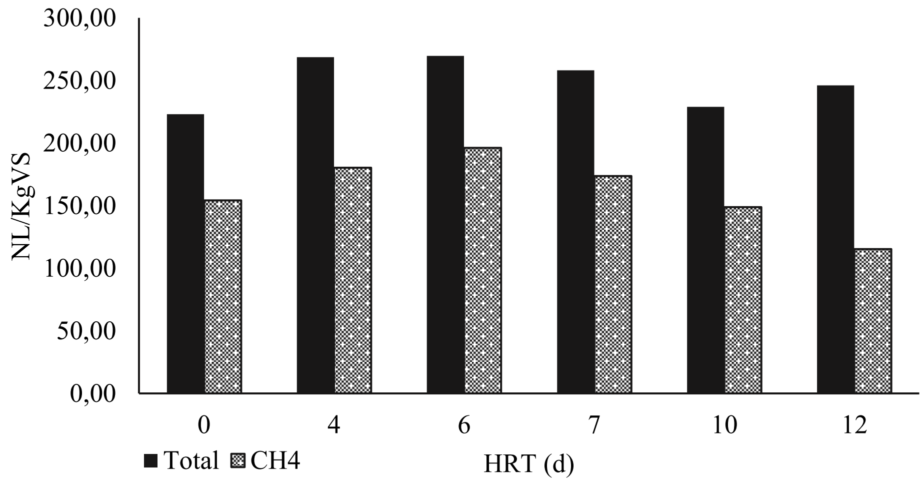

Figure 17. Graphical representation of the gaseous potential of the fermented outputs and fresh SMB (HRT 0 d).

Corn residue and Spoiled soybean meal

Corn residue fermentation was tested with three HRTs: 3 d, 5 d, and 7 d. Biohydrogen production was registered in each trial, achieving peaks of 18.5% v/v from HRT 3 d, 8.1% v/v from 5 d, and, 16.1% v/v from HRT 7 d. Different from the wheat straw fermentation, the highest biohydrogen concentration was revealed at the lower HRT (3 d), probably due to the presence of more biodegradable elements. In fact, as well as reaching a lower content in recalcitrant compounds, as lignin and hemicellulose (Table 13), corn residue also presents traces of grains, notoriously rich in starch (about 720 g/KgTS) [21].

Table 13. Cellulose, hemicellulose, and lignin content of wheat straw and corn residue

	Cellulose	Hemicellulose	Lignin	References
		g/kgTS		
Wheat straw	320-471	200-450	76-260	[22,23]
Corn residue	301-396	214-303	56-213	[24]

However, after about an HRT, in all cases, the processes started biomethane generation and biohydrogen was no longer detected. In particular, in Figure 19 are shown the trends of daily biohydrogen and biomethane production, coupled with outgoing pH trends. Initially, the switching of metabolism was attributed to the rise of VFAs concentration, at about 10 gCODvfa/L. A high content of acids could firstly decrease the pH of the medium below the DF range, and secondly, VFAs could trigger a feedback inhibition in acidogens. As visible in Figure 18, the protonated form of acids, generated at low pH, can easily move through the cell membrane and unbalance the inner pH, which is commonly neutral [16,25]. The microorganisms in a state of suffering are not able to grow and replicate, due to the use of energy (as ATP) to pump protons out of the membrane through the ATPase complex. Alternatively, the microorganism adopts the switching to an acid-consuming metabolism, such as methanogenesis or solventogenesis, as the strategy to survive [16,25].

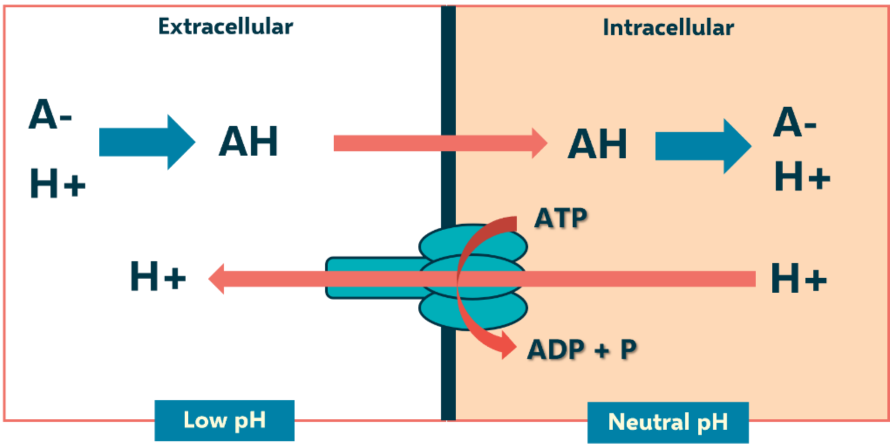

Figure 18. Representation of VFAs feedback inhibition.

For this motive, an integration of a semi-continuous buffer system in the HRT 3 d test was tested. A system composed of the use of digestate as a liquid part of the input. In particular, the output of DF was used as feed in the methanogenic phase reactor, and the digestate at about pH 7-8, once removed from the major part of methanogens, was recirculated in the first phase. The results showed, as reported in Figure 19, a peak of 19.5% v/v of biohydrogen and a time extension in its production of about twice the SRT value. Nevertheless, this strategy was not enough to reach the biohydrogen stability. Based on the corn residue composition, often in large-scale AD plants, co-digestion is applied to overcome the macro- and micro-element scarcity of the substrate. Furthermore, the hydrogenase enzymes need microelements as iron and nickel for the function of their active site and to produce biohydrogen [13–15]. For these reasons, the stop of biohydrogen production was attributed to a limitation in essential elements.

As a possible improvement of the process, a co-fermentation HRT 3 d with spoiled soybean meal was evaluated, due to its rich composition in nitrogen and micro-elements. In Table 14, the difference in trace elements (as Fe, Ni, Zn, and Cu) between corn residue and soybean meal is reported. As visible from the table below, soybean meal reports a higher content of micro-elements and nitrogen, which could provide more sustenance for the acidogenic community.

Table 14. Comparison between corn residue and soybean meal nitrogen and micro-elements (Fe, Ni, Cu, Zn) concentrations

	Fe	**Ni**	**Cu**	**Zn**	**N**	**Cit**
			mg/gTS			
Corn residue	0.099	0.004	0.004	0.029	60.0	[14,15]
Soybean Meal	0.235	0.006	0.065	0.054	310.8	[26,27]

In parallel, to have a comparison to the co-fermentation, an HRT 3 d test with only soybean residue as feed was developed. The biohydrogen and biomethane trends are reported in Figure 20 below and compared to the daily pH value. Both the tests with soybean residue reported a stable biohydrogen production, but in particular, the co-fermentation had the best SHP, of about 12.35 $NmLH_2/gVS$, compared to 4.71 $NmLH_2/gVS$ of mono-fermentation. From the results, the spoiled soybean meal contributed a sufficient amount of nutrients for the sustenance of acidogens and thus maintained stable biohydrogen generation. In addition, the combination of corn residue with the spoiled soybean meal improved the biohydrogen production through the increase of the C/N ratio from 2.9 to 5.1. Lastly, to confirm the stability achieved, no traces of biomethane were detected for both tests.

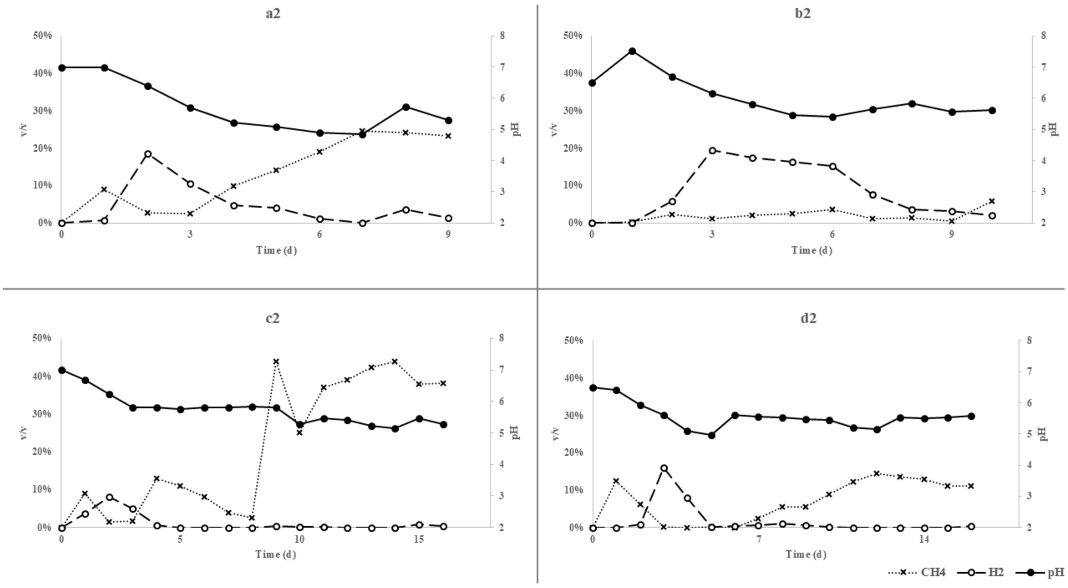

Figure 19. Corn residue gas and pH trends. HRT 3 d (a2), 3 d with recirculation system (b2), 5 d (c2) and 7 d (d2).

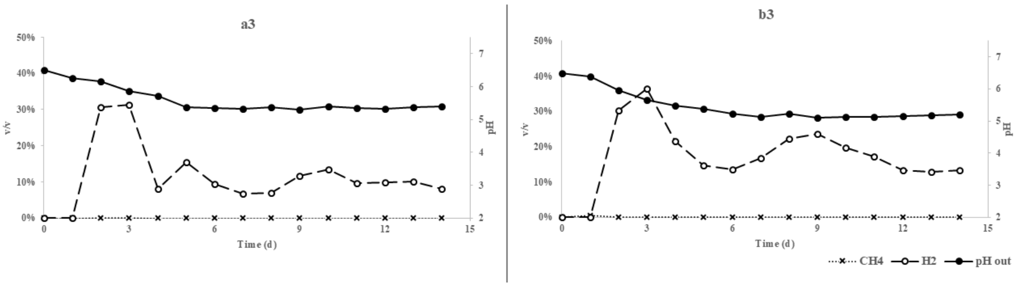

Figure 20. Hydrogen, methane and pH trends for soybean meal residue fermentation tests. HRT 3 d (a3) and HRT 3 d co-fermentation 50% VS with CRs (b3).

Regarding the VFAs production, the average concentration of each trial is reported in Figure 21 as gCODvfa/L. All the tests reported acetic and butyric generation, typical products of acidogenic fermentation [28,29]. To a lesser extent, propionic acid has also been found. Propionic acid production is often attributed to the conversion of lipids and proteins [18,19]. Fermentations registered also valeric acid and caproic acid production, compounds derived from local chain elongation phenomenon [30,31]. This metabolism is usually based on beta-oxidation reactions. In detail, two carbon atoms at a time (as acetyl-CoA) are added to the VFAs' hydrocarbon backbone. Several cycles can occur, leading to the formation of butyric acid, valeric acid, or caproic acid, depending on the starting compound and the medium conditions [31,32]. The reactions are driven by the employment of electron donors, for example, lactic acid, ethanol, hydrogen, or, in rare cases, also amino acids [32]. Given this, the production of medium-chain acids could be a cause of a reduction in biohydrogen detection. Alternatively, lactic acid may have been used, produced when the pH dropped below 5.5 (ideal condition for lactic fermentation) [33].

In yield terms, the best results were obtained from HRT 7 d with corn residue with 0.37 gCODvfa/gVS, followed by 0.33 gCODvfa/gVS (HRT 5 d corn residue), 0.32 gCODvfa/gVS (HRT 3 d co-fermentation soybean meal), 0.27 gCODvfa/gVS (HRT 3 d soybean meal), 0.27 gCODvfa/gVS (HRT 3 d recirculation corn residue) and, 0.26 gCODvfa/gVS (HRT 3 d corn residue). The best yield was achieved with the highest HRT with corn residue, probably due to the increased exposure time of the carbonaceous substrate to acidogenic conversion. As regards the production rate, the best result was achieved by HRT 3 d co-fermentation with spoiled soybean meal, with 5.1 gCODvfa/(L*d).

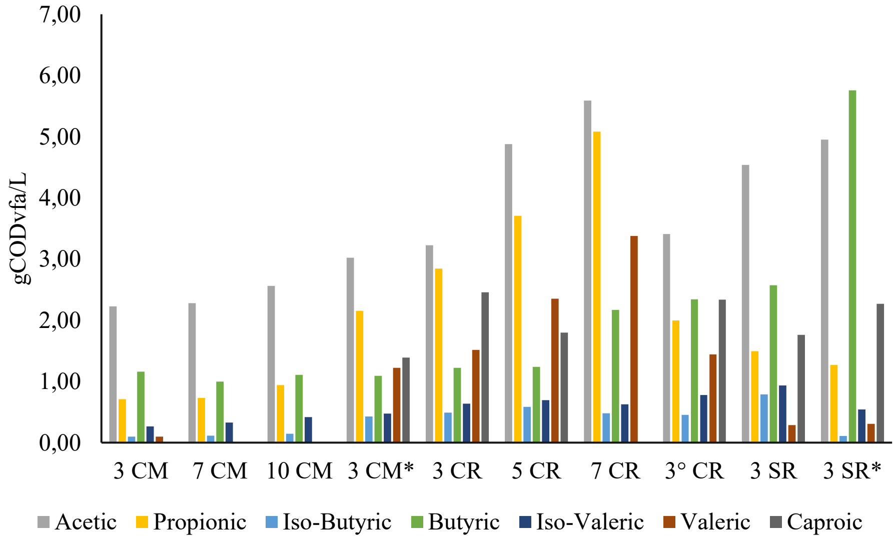

Figure 21. Charts of average VFAs concentration of each test. CR: corn residue; SR: spoiled soybean meal (co-fermentation; ° recirculation system)*

Once the fermentation outputs were collected, a metagenomic analysis was done on the last samples of the HRT 3 d tests (corn residue trial, soybean residue trial, corn residue with recirculation trial, co-fermentation trial). Traces of methanogens were found for mono-fermentation of corn residue, information that could explain the biomethane detected, and that permits assuming their presence also in higher HRT tests (5 d and 7 d), being more suitable for their kinetics. The recirculation test did not reveal methanogens' presence, although biomethane was identified on the last day of the analysis. In both corn-specific trials, mono-fermentation (24.93%) and recirculation (4.58%), *Veillonellaceae* were detected. Bacteria that consume lactate to produce VFA and caproic acid [34–37]. Also, *Prevotella*, propionate-producer genus was found, with a 4.54% and a 6.31% relative abundance [38]. Only the recirculation trial reported an *Acidaminococcus* presence (6.63%), recognized as a valeric acid and caproic acid producer [35]. All the samples analyzed reported an important amount of *Firmicutes*, microorganisms recognized for their biohydrogen-producing capabilities [39,40]. Some of the identified are members of *Clostridiaceae* (corn residue mono-fermentation trial 2.23% and recirculation trial 2.25%), *Lachnospiraceae* (corn residue recirculation trial 4.61%, soybean residue mono-fermentation trial 12.79% and co-fermentation trial 9.36%), and *Erysipelotrichaceae* (soybean residue co-fermentation trial 49.69%) [41–44]. Both the spoiled soybean meal tests reported the *Firmicutes'* highest presence, and in particular, in the mono-fermentation, they characterized the entire microbial community (about 99.4%). *Caproiciproducens* (*Ruminococcaceae* family) was also found (relative abundance lower than 3.0%), a microorganism that could be associated with the chain elongation and caproic acid presence [32,45]. Related to that, the soybean residue mono-fermentation also exposed the presence of *Streptococcus* (76.10%), bacteria able to produce lactic acid, the common intermediate of caproic production [46]. Finally, the employment of corn residue determined in every process the presence of *Bifidobacteriaceae* (mono-fermentation trial 45.92%, recirculation trial 56.82%, co-fermentation trial 27.49%), microorganisms capable of producing lactic acid and, as reported by Nishimura et al. (2018) [45], able to generate a positive effect for the biohydrogen production from DF.

From the remaining fermented outputs of corn residue mono-fermentations (HRT 3 d, HRT 5 d, and HRT 7 d), biomethanation potentials were evaluated and compared to the value of untreated feedstock. Two of the fermented outputs achieved better biomethane potentials than the fresh corn residue. In detail, the best improvement resulted from HRT 7 d output with 753.04 $NmLCH_4/gVS$, a +34.1% compared to the 561.76 $NmLCH_4/gVS$ of the untreated corn residue, followed by HRT 5 d with a more moderate +4.7% (588.32 $NmLCH_4/gVS$). The HRT 3 d fermented output reported the same result as fresh. As for cattle manure BMP tests, also, in this case, the efficiency of the “pre-treatment” of using a specific DF phase was verified, and in particular, reporting better hydrolyzations at long HRTs.

In conclusion, the lignocellulosic substrates (as SMB and corn residue) could be utilized for biohydrogen production in the DF process. However, some precautions are necessary to achieve the bioH_2 generation and to maintain the stability of the process. In particular, more attention should be given to HRT values and to the content of essential macro- and micro-elements.

Furthermore, the application of a specific DF reactor for an AD is further appealing, given its potential to increase the biomethane resulting yields.

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5. Economic analysis

5.1 Introduction

Taking into consideration what was reported in the previous chapters and based on the results obtained, an economic evaluation was developed for the implementation of a full scale TSAD process. The main goal of this exercise was to generate a preliminary consideration of the feasibility of the process, and the consequent possibility of having a profit.

Two study scenarios were assumed:

I. The entire plant construction (fermentation reactor, digester, and upgrading unit);
II. The integration of the fermentation stage from pre-existing biomethane plants.
Both developed within the agricultural context of agricultural residue reuse.

The evaluation was carried out on the basis of the results and parameters obtained from the literature, adopting a general perspective that excludes specific cases where parameter fluctuations, extraordinary maintenance, local variations in outdoor temperature, changes in feedstock input, and fluctuations in energy prices occur. The analysis was carried out taking into consideration the main expenses and sources of income. The expenses included the cost of building the plant and operating costs (temperature and mixing), while earnings were limitedly derived from the energy obtainable from the gaseous fuels. In the next section, the methodologies are clearly outlined.

5.2. Materials and Methods

The economic evaluation was based on the data acquired during the fermentation and potential biomethanation tests, considering the full-scale integration of the plant and its results over the years. The analysis was limited to the cost derived from the implementation of the facilities and energy used to maintain the parameters (temperature, mixing). Any additional expenses not directly related to the use of the reactor were excluded, such as land purchase, personnel, and feedstock transportation. In fact, it was assumed that the plant was built directly within a farm. And that the dedicated workers were the same as those employed in the other agricultural tasks. As for earnings, the sale of energy derived from the fuels generated was considered, excluding any savings in the use of digestate as fertilizer and any European or national monetary incentive.

Moreover, CSTR-type reactors like those typically used in the rural scenario were considered. Both for the first and second scenarios has been taken into consideration a digestion reactor of medium size of 5500 m^3, with an operative volume of 5000 m^3. The HRT chosen was 50 days, the same as used for the BMP tests. A working concentration of 5% in TS was imposed for consistency with fermentation tests. The reactor was not assumed to have a pH control system. The temperature was maintained at 38°C through heating systems, and the feed was constantly mixed with a power factor (*Pf*) of 0.01 $kW*m^3$. It was assumed that the reactor was constructed of concrete and coated with isolation panels, with a limited transfer coefficient of 0.01 $W/m^2°C$. As for the fermentation reactor, it was built consistently with the digester, keeping the same heating system, agitation, and construction materials. Equipped with a working volume of 300 m^3 (in a total of 500 m^3), with a HRT of 3 days, and limited in TS content of 5%. Also, in the DF reactor, a pH control system was not included. The DF reactor in the design of the experiment was placed upstream of the digestion process, consistent with the TSAD process. In addition, it was considered a plant including an upgrading unit for the cleaning of biogas generated. The substrate chosen for economic evaluation was a mixture of 50% -50% VS mix corn residue and soybean residue. So that the feedstock was as similar as possible to that used in the fermentation test that achieved continuous biohydrogen generation. As reported in Paragraph 4.3.

Installation cost

The cost for the plant construction was calculated following the equation below, taking into account the price per m^3 for the actual reactor volume. The estimated price was equivalent to 135.2 €/m^3, based on what was reported by Sahoo et al. (2019) [1] for liquid-state reactors [2], and weighted according to cost increases [3].

$$Installation\ cost\ (€) = Price\ per\ volume\ \left(\frac{€}{m^3}\right) \times Volume\ (m^3) \qquad \text{(Eq.15)}$$

Biogas upgrade unit costs

The costs derived from the upgrading unit are due to its installation and the treatment performed on the volume of biogas input. A unit with Pressure Swing Adsorption (PSA) technology with a maximum capacity of 600 m^3/h was considered, with an estimated price of 2.19 million euros [3,4]. Capable of working at a price of 0.19 €/m^3 (with 65% methane in biogas). Given that, upgrading systems for biogas containing biohydrogen have not yet been employed, so the same unit was assumed to be used.

Heating cost

The reactors were integrated with a heating and temperature maintenance system, which had been set at 38°C. The energy expenditure to maintain this parameter is significant, being derived primarily from heating the feed and then counteracting heat loss, estimating an average outdoor temperature of about 11°C. According to Garkoti et al. (2024) [5], Equation 16 permits to evaluate the energy required to heat the substrate, while Equation 17 is used to estimate the energy loss and thus the equivalent required to maintain the desired operating conditions.

$$Q = m \times Cp \times \Delta T \quad \text{(Eq.16)}$$

With specific reference to Equation 16, the heat required Q (J/d) was calculated from the mathematical product of the mass flow rate of the influent m (kg/d), the specific heat of influent Cp (J/(kg*K)), and the difference between the internal and feed temperatures ΔT (K).

$$H = Uo \times A \times \Delta T \quad \text{(Eq.17)}$$

Specifically in Equation 17, the product of heat transfer coefficient Uo (W/(m^2*K)), the wall area exposed A (m^2), and the difference between the internal and external temperatures ΔT (K), was used to calculate the rate of heat loss H (W).

Mixing costs

The reactors employed were CSTR-type, thus continuously mixed. They were equipped with mixing systems aimed at homogenizing the mixture to promote microbial contact with the organic matter and facilitate degassing, generating new passages through the material to recover biogas bubbles. According to Arango-Osorio et al. (2019) [6], the energy demand for mixing a reactor can be estimated according to Equation 18 below. The mixer power Wr (kW) was calculated from the Pf (0.1-0.01 kW/m^3) and the operative volume Vop (m^3) of the reactor.

$$Wr = Pf \times Vop \qquad \text{(Eq.18)}$$

Fuels profit

The economic revenue of the process was estimated from the sale of the energy generated from the biomethane and biohydrogen gases, obtainable under optimal conditions. The estimate was based on the heating value of biomethane (49.8 kJ/Kg) and biohydrogen (119.75 kJ/Kg), then was converted to kWh so it was possible to relate it to an economic return (€), calculated with the average European energy price of 0.22 €/kWh [7]. The amount of green fuels generated was calculated based on the product of the organic matter entering the process (t VS) and the yield values (m^3gas/t VS) obtained from this study and from the literature [8]. Also, taking into consideration the loss of biomethane and biohydrogen of 2.96% v/v [9] due to the upgrading step.

5.3. Results and Discussion

The economic evaluation was carried out taking into consideration what was reported in the previous paragraph. The results were projected over the years, with a view to assessing whether it was possible to amortize the costs, and possibly for how long.

In the table below, a summary of the results is reported for each category.

Table 15. Details of expenses and revenues of the two scenarios

	Scenario 1	**Scenario 2**
	Outflows	
	k€	
Reactors	811.3	67.6
Upgrading unit	2189.5	0.0
	k€/y	
Heating	231.5	231.5
Thermal loss	230.2	230.2
Mixing	102.1	102.1
Upgrading	330.3	330.3
	Inflows	
	k€/y	
Biomethane	1773.8	1773.8
Biohydrogen	14.0	14.0

As reported in Scenario 1 of Table 15, the construction from scratch of the plant (reactors and upgrading) is what had the greatest impact on the expenses for a TSAD process. As for annual expenses, however, the cost of biogas upgrading was the most impactful. This shows the importance of an efficient upgrading system in order to make the process sustainable. In addition, the second highest expenses were the heating and thermal maintenance of the reactor. In particular, the use of cement alone for construction and the relative absence of insulation material make the expenses unsustainable, due to a heat transfer coefficient of about 2 W/m^2K.

In the figure below, the economic balances of the process in the two scenarios spread over 10 years are depicted.

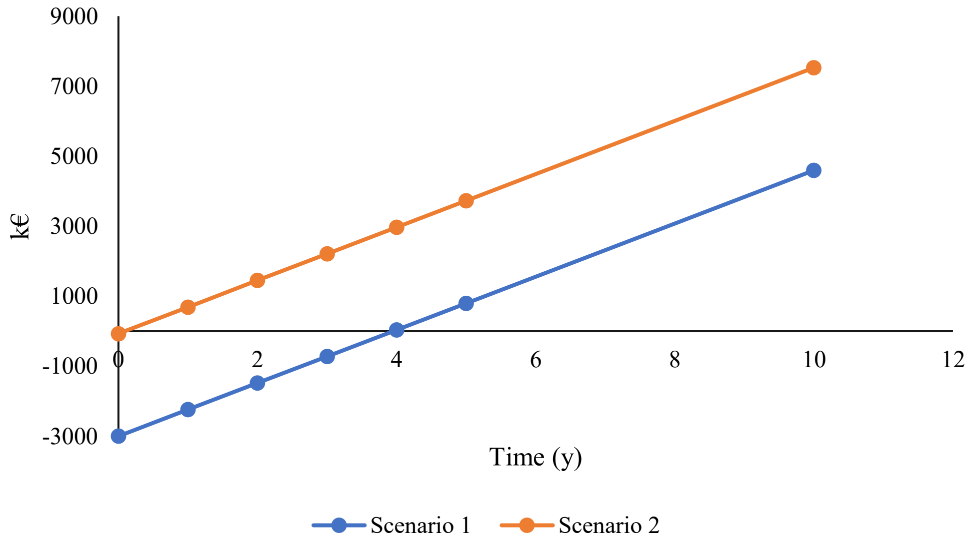

Figure 22. Estimated earnings over the years (Scenario 1 and 2)

The long-term economic analysis allows to estimate the sustainability of the integration of the TSAD process in the two scenarios. Regarding the first, at least 4 years are required to pay back the investment expenses. During which additional expenses, for example, due to maintenance, are unlikely to be avoided. The estimation worked differently for the second scenario. The absence of expenses for the construction of the digester and upgrading unit makes it possible to pay back the costs within about a year. Furthermore, the integration of this process, after a period of about 13 years could start to bring higher gains compared to the use of traditional single stage AD plants for biomethane production. Achieving in 20 years an increase in earnings of about 36,130 €.

However, it must be kept well in mind that this economic evaluation is based on the results of this study, carried out on a limited scale, such as a laboratory scale. Moreover, considering literature parameters that may not always coincide with different real situations, in which, for example, the input feed may vary seasonally. But nevertheless, this assessment can help to make some considerations about the process. Such as considering partial integration into existing digestion and upgrading plants (Scenario 2) more feasible.

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6. Conclusions

In the previous chapters of this thesis, the TSAD process was discussed in detail, drawing out its strengths and weaknesses. A possible evolution of the traditional AD process that can reduce the carbon footprint of the resulting injectable fuel (biohythane) in the natural gas grid, consistent with European decarbonization targets. The investigation was focused on the application of agricultural residues, lignocellulosic matrices produced in high quantities and often not completely revalorized.

The experimental part of the thesis begins by evaluating the usefulness of some pretreatments as an approach to increase process efficiency. However, obtaining results that do not justify this type of integration. The thesis continues by evaluating animal-derived matrices (manures and slurries) as substrates for the TSAD process. Matrices that seem to be able to allow the separation of the two metabolic phases, appreciably increasing biomethane yields, but are unlikely to find use in biohydrogen production. Highlighting how much the presence of methanogens in the feedstocks is too limiting, except under certain co-fermentation conditions. Then the study addresses feedstocks of plant origin, which report much more encouraging results in biohydrogen generation, but which need some precautions and strategies to achieve continuous production. The control of pH and the presence of macro- and micronutrients are essential in maintaining the stability of the fermentation phase. From the study of the different matrices, emerged the importance of using specific HRTs that can best support the various stages of the process.

Following the results obtained from the experimental tests, an economic evaluation of the possible implementation of the process on a full scale is reported. Analysis was carried out on the basis of the results obtained and according to references found in the literature. Two scenarios were evaluated: the entire construction of the plant and only the integration of the fermentation stage. The evaluation concludes by showing the possibility of amortizing costs in both scenarios, but with markedly different timelines that make integration alone on pre-existing plants more economically attractive.

This study shows how the TSAD process from agricultural residues appears to be promising in making anaerobic digestion plants and gas grid more sustainable. Two phases separation has also been shown to be useful in boosting VFA production, with circumscribed chain elongation phenomena, creating a possible secondary utility of the process. In anticipation of real implementation, these results must also be validated on a larger scale, such as at the pilot scale. In addition, regulations will need to be adjusted to justify the investment, as at present the commercial value of hydrogen is mainly recognized in its pure form and not as a blend injectable in the gas grid.

7. Addendum

7.1. Introduction

During the six-month period abroad at the School of Engineering at the University of Aberdeen (Scotland, UK) in collaboration with Prof. Davide Dionisi, an in-depth study of the use of SBRs was undertaken.

The efficiency of the AD and TSAD processes is determined by many factors such as the type of substrate, the operating parameters used, and the microbial communities involved. However, the type of reactor employed can also be a key element of the process and can vary depending on the desired use. SBR reactors are especially employed in wastewater treatment processes to have complete degradation of organic material and consecutive clarification of the medium. The SBR is a fill-and-draw reactor, based on multiple cycles in the same vessel, involving: I. Filling with new feed, II. Reaction with microbial biomass under mixing conditions, III. Settling of solids, IV. Settling of supernatant, and V. Emptying of the clarified portion.

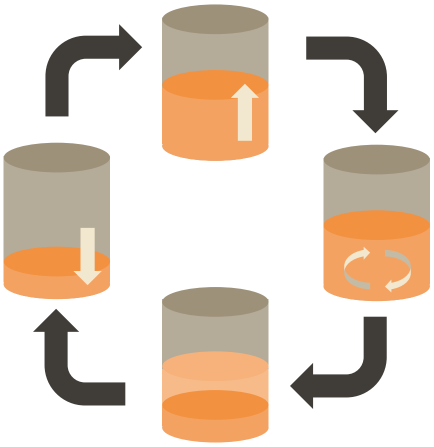


Figure 23. Schematic diagram of SBR reactor operation

This technology allows for the decoupling of HRT and SRT parameters. Working under the condition of low HRT and high SRT allows operating on high inlet volumes without the risk of biomass washout while being able to achieve better degradation of particulate material.

The characteristics of the SBR reactor would seem to fully comply with the ideal parameters of DF, which requires working at high rates with low HRT and high OLR. It also succeeds in overcoming the limitation of the washout of the microbial community of a CSTR reactor.

For these reasons, it was chosen to verify the SBRs application on the TSAD process. To do so, it was decided to limit the difficulties of using a recalcitrant substrate such as agricultural residues by employing a synthetic feedstock of OFMSW.

7.2. Materials and Methods

The analysis was divided into three steps: synthetic feedstock preparation, substrate and inoculum characterization, and TSAD testing in SBR reactors. The feedstock was prepared according to the formulation reported by Simonetti et al. (2023) [1], a composition that mimics the elements (fats, proteins, fiber, total carbohydrates, and sugars) present in real OFMSW (Table 16). Once the substrate was prepared, agricultural digestate from "John Rennie & Sons, Gask Farm" agricultural company (Turriff, Aberdeenshire) was taken as a source of acidogenic and methanogenic microorganisms. Substrate and inoculum were characterized in terms of TS, VS, total suspended solids (TSS), total volatile suspended solids (TVSS), COD, and soluble COD (sCOD). Analysis of TS and VS was carried out by the gravimetric method on ceramic crucibles, following heating of the sample first at 105 °C in an oven for removal of the aqueous component, and then at 550 °C in a muffle furnace for ashing. The same method was used for TSS and TVSS, but using 0.2 µm filters. "Hack" spectrophotometric kits were used for the evaluation of COD, and sCOD after specific sample filtration.

Table 16. Synthetic feed composition

Synthetic OFMSW	
	g/L
Wheat grass powder	9.0
Starch	10.0
Peptone	5.8
Yeast Extract	3.3
Sacrose	8.3
Oleic Acid	6.5

Once the feed and inoculum characteristics were obtained, analysis could be started in SBR reactors. The TSAD process was organized into five glass vessels, connected in series, and kept sealed to prevent gas leakage. In detail, in Figure 24 are represented from left to right: feed bottle, DF SBR reactor, DF efflux bottle, methanogenic SBR reactor, and final efflux bottle. The two SBR reactors were equipped with a 1 L working volume and a 38 °C heating system using a thermal bath. The pHs were controlled daily through the addition of sodium hydroxide and sodium bicarbonate, keeping the two phases in the optimal range of 5.5-6.5 and 6.5-8.0 for DF and methanogenesis, respectively. The glass vessels were connected in series through the use of peristaltic pumps, kept mixing by magnetic stirrers, and equipped with gas quantification ("Ritter MilliGascounters") and gas collection systems (gas bags). The setup was organized with timers to ensure proper timing for settling the solids and emptying the clarified liquid. For SBRs, two daily cycles were used for the emptying and filling phases. The acidogenic and methanogenic phases operated at HRT 2 d (OLR of 31.21±1.5 gCOD/(L*d)) and 6 d (OLR of 7.9±2.2 gCOD/(L*d)), respectively. It was not possible to extend the range of difference between the two HRT values, given the limitations of reactor volumes and the range of peristaltic pumps.

Figure 24. Setup of TSAD process.

The analysis was performed for a total of 110 days. The process was started by adding a 5% v/v aliquot of digestate (diluted in water) to the two SBR reactors to ensure inoculation. Once the trial started, liquid and gaseous samples were collected daily to evaluate: VFA content (through "Thermo Fisher UHPLC"), solids content, COD content, gas production, and gas content (using "Thermo Scientific Trace 1300 GC-TCD" and "Geotech Biogas 5000 analyzer"). Checks carried out to evaluate the progress of the process. Weekly, the actual SRT was calculated for each process and then compared with the HRT value.

7.3. Results and Discussion

Once the feed was prepared and the agricultural digestate collected, they were characterized, and the results are shown in the table below. The resulting VS/TS ratio of the synthetic feed was 94.8% w/w, a value that highlights that the substrate was not fully biodegradable due to the use of tap water for preparation and sodium bicarbonate to adjust the pH value. In addition, only 1/3 of the COD present was in soluble form, thus immediately accessible to microbial attack.

Table 17. Characterization of agricultural digestate and synthetic feed

	TS	**VS**	**COD**	**sCOD**
	% w/w	% w/w	g/L	g/L
Agricultural Digestate	4.5	3.3	53.4	12.7
Synthetic feed	3.84±0.12	3.61±0.06	62.42±3.08	23.86±2.49

As previously reported, the values of TSS and TVSS, which were used to calculate the actual SRTs, were also monitored during experimentation. As shown in Table 18 and Figure 25, the TSS and TVSS content decreases as the substrate proceeds in the vessels. The final effluent reports a degradation of TVSS of about 76.4% w/w compared to the starting feed; in addition, TSS was also reduced by about 66.9%

w/w, due to the sedimentation and clarification system of the SBRs. Through these values, it was possible to calculate the effective SRTs, which were 4.1 d and 22.2 d for the DF and methanogenic phase, respectively. These values showed that the process may have been set up correctly, as it was able to retain the part not yet clarified and rich in solids. Specifically, extending the SRT value by 2.1 times compared to the HRT of the DF and by 3.7 times for the methanogenesis step.

Table 18. TSS, TVSS, TVSS/TSS ratio of each step of the process

	Feed Tank	**First SBR**	**First effluent**	**Second SBR**	**Final effluent**
TSS%	3.05	2.74	1.34	1.22	1.01
TVSS%	3.05	2.68	1.31	0.98	0.72
TVSS/TSS%	100.0	97.8	97.7	80.3	71.3

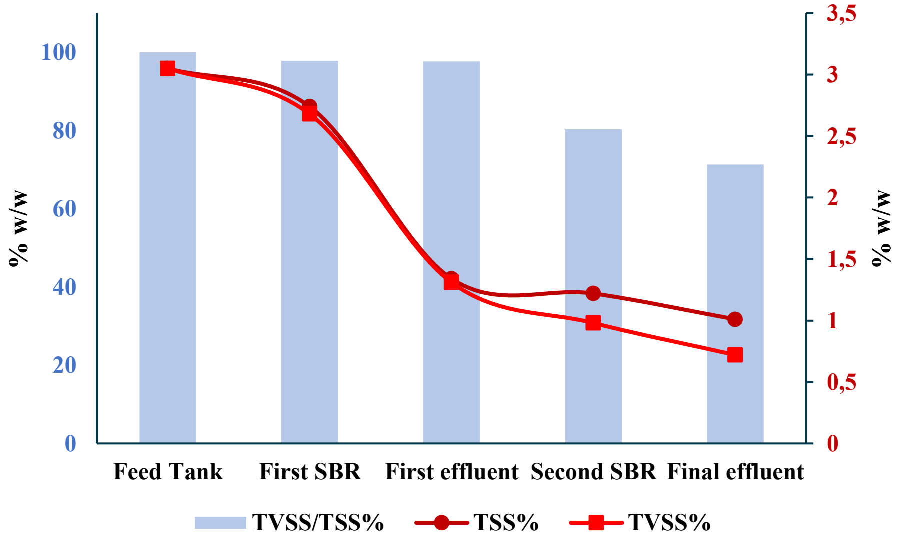


Figure 25. Average TSS, TVSS and TVSS/TSS values at each stage of the process

Daily, in the course of the analysis, the content of VFAs, compounds produced specifically by the fermentation stage, was evaluated. At the end of the test, it was thus possible to determine the conversion yield of the process (following Equation 10 but using daily COD input as the denominator). The results showed a VFAs yield of 16.2% (gCODvfa/gCOD) from the DF phase and 19.6% from the whole process. Contrary to what was hoped for, there was also an increase in VFA concentration in the second phase as well, with a yield of 4.4% (gCODvfa/gCOD). Metabolic phase in which VFAs should be converted to CH_4 and CO_2, demonstrating a decrease in concentration in the medium. The reason was attributed to the difficulty of maintaining the pH within the ideal ranges of methanogenesis (>6.5), which may have partially inhibited the process.

During both phases, mainly short-chain VFAs were produced, particularly acetic acid, propionic acid, and butyric acid. But traces of iso-valeric and valeric acids were also found. Table 19 below shows the concentrations of VFAs in detail.

Table 19. VFAs content of first and second effluent

	Acetic	Propionic	(Iso-)Butyric	Iso-Valeric	Valeric
			$\frac{gCOD}{L}$		
First phase	3.3	2.7	1.5	1.2	1.4
Second phase	5.6	3.5	1.9	0.3	0.9

The presence of acetic and butyric acid is typical of the acidogenic fermentation of carbohydrates, while propionic acid is attributed to protein and lipid conversion.

The reduced presence of valeric and iso-valeric may have resulted from local chain elongation phenomena [2,3]. Reaction triggered by the presence of electron donors such as lactic acid, ethanol, hydrogen, or amino acids. In this regard, lactic acid was found during the DF stage, in particular at the lowering of pH below 6.0. The average lactic acid concentration was 1.4 gCOD/L. As reported in Figure 26, the finding of traces of lactic acid corresponds to the decreases in pH, furthermore, its content decreases as valeric acid increases. Phenomenon respecting a possible chain elongation metabolism [2,3].

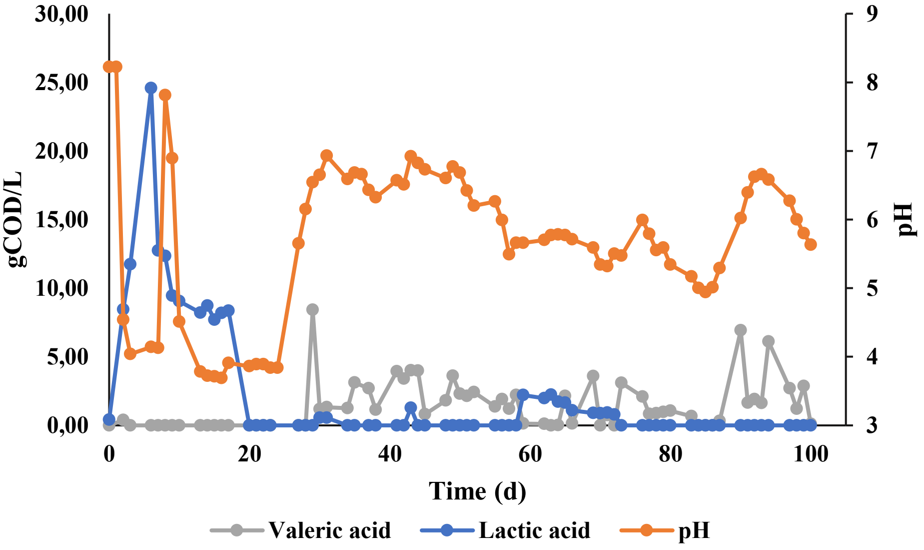
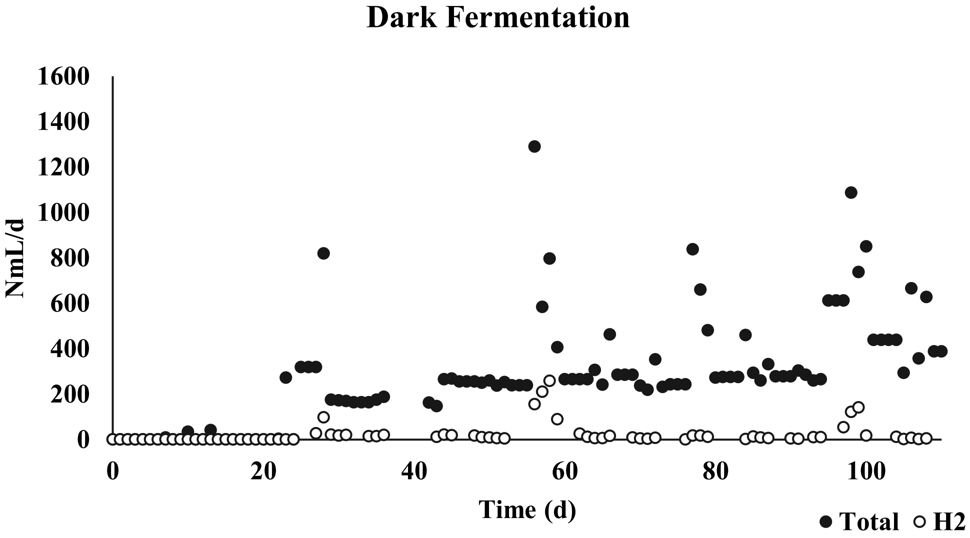


Figure 26. Valeric acid, lactic acid and pH trends of DF phase.

Regarding the gas analysis, the total gas and biohydrogen content of the first stage was evaluated, and in the second stage, also that of biomethane. In detail, the daily gas production for the first and second phases is reported in Figure 27. In a few days, the first reactor reported biohydrogen production, a sign that the DF phase had been reached. Production that could not be kept constant during the test, but can be roughly summarized as a yield of 0.87±0.25 $NLH_2/KgCOD$ (calculated with Equation 8), with peaks of 36.0% v/v. The factor that may have been the cause of the instability and that reported the most problems was the pH of the medium. Even through the daily addition of alkaline compounds, maintaining the pH within optimal values was difficult. Initially attempted with the use of NaOH, it then had to be converted to the use of sodium bicarbonate as a buffering agent. The use of the buffer improved pH control, but did not achieve satisfactory results. While concerning the methanogenic gas analysis, a biomethane production was reported with a yield of 5.02±0.61 $NLCH_4/KgCOD$ (calculated with Equation 7) and peaks of 34.7% v/v. The results for biomethane were also below expectations [4], in fact, better results can be found in the literature, as Sarkar et al. (2021) [5] achieved a biohydrogen yield of 96 $LH_2/KgCOD$ and a biomethane yield of 117 $LCH_4/KgCOD$ from real OFMSW in laboratory batch tests, or Yeshanew et al. (2016) [6] reported yields of 115 $LH_2/KgVS$ and 335 $LCH_4/KgCOD$ from food waste in an integrated system of CSTR and anaerobic fixed bed reactors (AFBRs).

The reasons were probably the pH control problems, as for the DF, and the HRT employed, which was lower than the methanogenesis optimum (>15 d) [4]. The process in its entirety, mixing the resulting gases, achieved a biohythane yield of 5.89 NL/KgCOD, consisting of 14.8% v/v biohydrogen and 85.2% v/v biomethane.



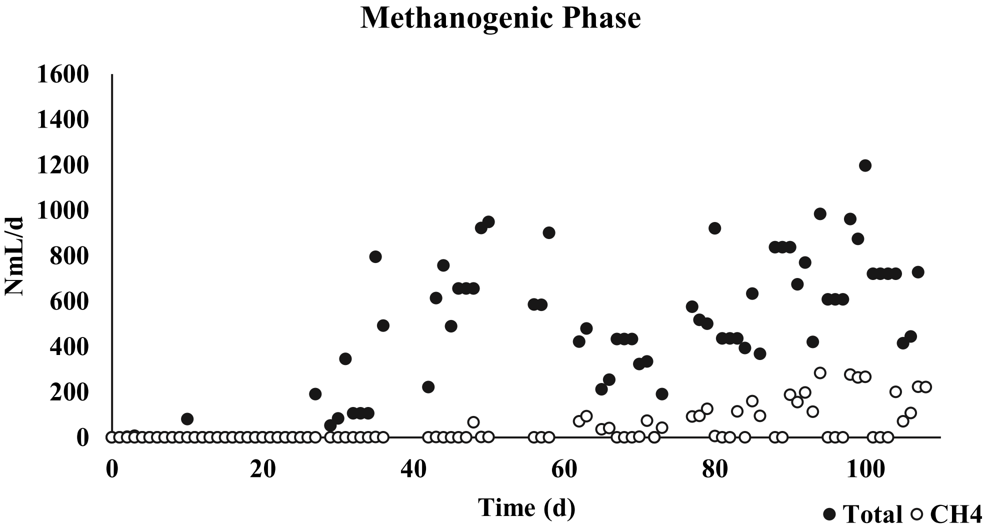


Figure 27. Daily gas productions of the DF and methanogenic phase

Following the production of biohydrogen, VFA, and biomethane, the separation of the two AD phases using SBR reactors appears feasible, at least on a laboratory scale. From the data collected from this analysis, intending to scale up the process, the need for further studies is evident. In that, both steps must be optimized for achieving competitive yields compared to what has been achieved in the literature using other types of reactors. The adoption of different substrates could also allow avoiding the problem of difficult maintenance of pH values. Although the results in gas and VFA were not at the level of the literature, the process still reported the successful decoupling of HRT and SRT parameters. And it also achieved the abatement of organic matter in the stream by 76% w/w.

7.4. References

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