Università degli Studi della Tuscia di Viterbo

Dipartimento di Scienze Ecologiche e Biologiche

Corso di Dottorato di Ricerca in

Ecologia e gestione sostenibile delle risorse ambientali - XXXII ciclo

"BIOLOGICAL BIOGAS UPGRADING TO BIOMETHANE : EVOLUTION OF METHANE PRODUCTION MICROBIAL COMMUNITY IN RESPONSE TO HYDROGEN ADDITION "

Settore scientifico-disciplinare

BIO/07

Tesi di dottorato di:

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A.A. 2020/21

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Research group during European Researchers’ Night at ENEA.

Outline

This thesis is composed as follows: a preliminary general introduction and state of the art review. Then the work is developed through a collection of two articles; finally, a chapter with general conclusions is presented. Titles of the papers that constitute this thesis are listed below:

- Lembo, G., Rosa, S., Miritana, V.M., Marone, A., Massini, G., Fenice, M., Signorini, A., 2021. Thermophilic anaerobic digestion of second cheese whey: Microbial community response to H_2 addition in a partially immobilized anaerobic hybrid reactor. Processes 9, 1– 16.
- Lembo, G., Rosa, S., Petrazzuolo F., Massini, G., Fenice, M., Signorini, A., 2021. *In situ* biogas upgrading in partially immobilized anaerobic hybrid reactor. Paper in submission

Summary and general purpose

Energy from biomass is considered throughout the world as a type of sustainable and renewable energy. In particular, the anaerobic digestion (AD) of biomass produces a biogas with a content of about 50-60% of CH_4 and 40-50% of CO_2. However, the upgrading of biogas with > 90% of CH_4 content has a higher calorific value, and it can be directly injected into the national grid or directly used as fuel by methane-powered vehicles.

Currently, physical-chemical methods for the upgrading of biogas to biomethane are already commercially used in the biogas industry. However these technologies increase biogas production costs by 20-72% due to high investment, electricity demand and chemical-water requirement. Moreover the CO_2 is release into the atmosphere.

An attractive and cost effective method to increase the CH_4 content in biogas is based on the biological reduction of CO_2 to CH_4 by using H_2 produced either biologically or through water electrolysis using off-peak electricity surplus from wind or photovoltaic plants. In the AD process this reaction is carried out by hydrogenotrophic methanogens.

Currently two main process approaches are used for the biological methanation: the *in situ* methanation, which consists of the direct injection of H_2 into the anaerobic digester, using internally produced CO_2 and the *ex situ* methanation, realized into a separate reactor where H_2 and CO_2 or H_2 and biogas are injected and containing enriched cultures of hydrogenotrophic methanogens. Both biological upgrading technologies are still under developing.

In particular, *in situ* biomethantion research was developed with the aim to modify the existing full-scale biogas plants, reducing the initial investment cost and the plant operation complexity. Among

biogas plant the continuously stirring reactor (CSTR) configuration is the most popular employed for anaerobic treatment of industrial, domestic and municipal wastes.

Several studies suggest that the H_2 diffusion in the liquid phase is the limiting factor of the process since the low H_2 solubility make it not bioavailable for microorganisms. In order to overcome this limit, several *in situ* strategies for CSTR reactor configuration have been proposed, mainly, introducing different H_2 injection devices, all using different stirring speeds or biogas recirculation rates. Although the hallow fibre membrane plus stirring seems to be the most favorable candidate for *in situ* biomethanation, the biofilm formation on the membrane damages the permeability and the lifespan of the membrane itself and then the connected costs have to be considered for full-scale applications.

To overcome this problem, the project PhD course proposes the immobilization strategy as a method to extending gas-liquid contact area as well as to increase microorganisms abundance. Then a new anaerobic hybrid reactor, defined as Gas Stirred Tank Reactor (GSTR) where about one third of the reactor working volume is filled with polymeric supports (high-density polyethylene, HDPE) was developed. The biogas recirculation was also applied to ensuring proper mixing of substrate and nutrients within the reactor and to increasing the availability of H_2 for the hydrogenotrophic community. Moreover to counteract the increase of pH due to removal of CO_2, an easily fermentable substrate was chosen, such as second cheese whey, a by-product of the dairy industry, very abundant in Italy and in Europe.

Nevertheless the direct injection of H_2 into the reactor brings various implications that shape the microbial community and the stability of the entire AD process. The increase of dissolved H_2 affects all the bacterial guilds that use hydrogen in their metabolic pathways. These microorganisms include hydrogen-producing guilds such as acid-forming and acetogenic bacteria, as well as bacteria that consume hydrogen, such as homoacetogenic and hydrogenotrophic methanogenic bacteria. In this way the entire AD trophic chain is exposed to altered conditions and the system is put under stress.

It is therefore clear that a deeper knowledge and characterization of the structure of the microbial community involved in biogas upgrading systems will provide useful information for the optimization of the process.

For this purpose, the community structure was monitored using Next Generation Sequencing (NGS) technique. The extracted DNA was sequenced on the Illumina platform. Further investigations on the structure of the microbial community were carried out using the FISH

(Fluorescence *In Situ* Hybridization) technique in order to evaluate the relationship between the Bacteria and Archaea domains. Samples were collected inside the reactor to investigate the microbial community response to H_2 addition on both immobilized area and effluent.

Finally, the potential of the GSTR configuration to enhance H_2 consuming efficiency by increasing gas recirculation rates and CO_2/H_2 ratio was investigated. The response of microbial community structure was studied by NGS techniques on effluent.

1 Introduction

1.1 Renewable Energy background

The demand for electricity globally continues to increase, since the latest report by BP, (British Petroleum Report, 2018) it is noted that in 2018 the global energy requirement grew by 2.9%.This ever increasing energy demand is strongly correlated to an ever greater economic development and, at the same time, to an increase in greenhouse gas emissions (GHGs). The same report notes that approximately 86% of the world's energy needs are met by the use of fossil fuels such as oil, coal and natural gas. The main contribution to energy consumption comes from countries that have been experiencing strong economic development in the last 10 years such as China and India.

But even in the countries of the European Union, the dominant energy source remains fossil fuel, even if the share of renewable energy is slightly increasing from 7% in 2016 to 8% in 2017.The increase in the use of renewable energy is mainly due to the expansion of wind, solar and solid biofuels. The share of gross final consumption of energy from renewable sources reached 19% in the European Union (EU28) in 2019, more than doubling the share of 2004 (8.5%). Furthermore, among the 28 EU Member States, eleven have already reached their national targets for 2020; Italy included (17.4 out of a 17% target) (Figure 1).

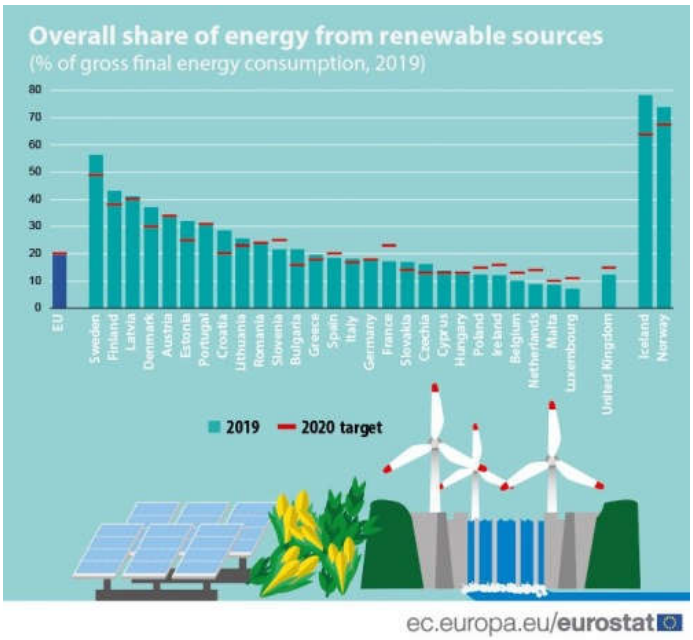


Figure 1. Share of renewable energies in gross final energy (ec.europe.eu/eurostat).

The European Union (EU27) in 2018 established further increase the binding target from 27% to

at least 32% share of renewable energy by 2030. More in detail:

First generation biofuels, based on food crop, must be limited to 2020 target (maximum 1% more) and it did not have exceed 7% of the final consumption of road and rail transport.

- The share of advanced biofuels and biogas must be at least 1% in 2025 and at least 3.5% in 2030.

Within this European program plan, each country must define its own integrated national plans for energy and climate. (PNIEC)
In Italy, the PNIEC, recently submitted to European Union, foresees the coverage in 2030 of 30% of gross final energy consumption with renewable source, in line with European target. Such a target will have to be achieved by expanding all source, both for electricity generation and thermal and transport uses.

1.2 Energy from biomass

Energy from biomass (or bioenergy) can meet the demand for renewable energy in any form. However, transport is the sector of most significant interest with correspondingly more excellent growth prospects, since the demand for biofuels, and particularly advanced biofuels (obtained from residual biomass and organic waste not in competition for land use with agricultural production for food or animal feed use), will progressively grow.

Currently a wide spectrum of technologies is used to transform biomass into bio-fuels, solid, liquid or gaseous and / or substances with high added value. All these technologies are based on physical, chemical and biological processes or a combination of them.

Figure 2 shows a flow chart of the conversion processes and their main products.

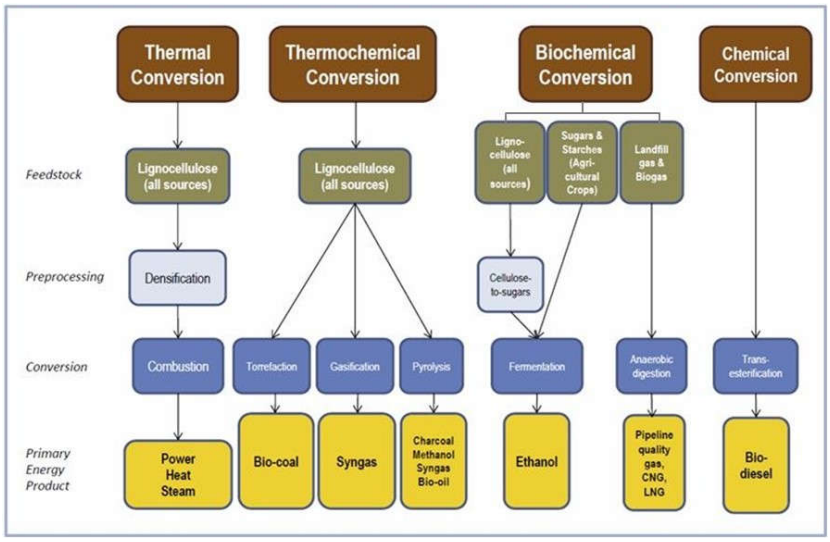


Figure 2. Biomass conversion processes flow-chart.

Among biological processes, anaerobic digestion (AD) can use a wide variety of organic waste to produce biogas, a gas composed of a mixture of CH_4 and CO_2

AD was initially used for the treatment and the stabilization of sewage sludge. It is currently mainly used for the treatment of livestock residues, agricultural crop residues, and dedicated crops known as energy crops. In addition to the production of methane, AD produces the digestate, an effluent that can be spread on agricultural land as a soil conditioner (Guebitz et al., 2015).

Compared to thermo-chemical technologies, AD is much more flexible in terms of usable raw materials and is considered more efficient and economically viable (Sarker et al., 2018)

One of the main disadvantages of the biogas produced from AD is the dilution of CH_4 with CO_2 and with traces of other gases such as H_2S, N_2, O_2, H_2O and siloxanes (Angelidaki et al., 2019). These results lower the calorific value of the biogas (Awe et al., 2017; Sun et al., 2015).

Most AD plants typically burn biogas in a Combined Heat and Power unit (CHP), producing thermal and electric energy. Alternatively, the produced biogas can be purified into a gas comparable to natural gas, which can be directly injected into the existing national grid or be used directly as fuel for vehicles. To obtain methane-rich biogas it is necessary to remove the CO_2, thus obtaining the so-called “biomethane” (Kougias et al., 2017).

The specific requirements for direct injection of biomethane into natural gas networks or its exploitation as a vehicle fuel, vary across countries and over a wide range of composition, such as:

CH_4 80-96%, CO_2 2-3%, O_2 0.2-0.5% (Muñoz et al., 2015; Patterson et al., 2013). During this last decade, biogas purification as biomethane transformation represented a huge opportunity to increase energy recovery from different types of waste and wastewater matrices. In 2017, biomethane production reached 19,352 GWh, increasing 12% from the previous year alone (European Biogas Association, 2018).

To date, there are about five hundred biomethane plants in European countries, mostly located in Germany (192), the United Kingdom (85) and Sweden (63). In Italy four plants produce biomethane from the organic fraction of municipal solid waste (FORSU), while three plants are under construction and will soon come into function (Etra - Padova PD, Acea Pinerolese - Pinerolo TO; Asja / VUS - Foligno PG) (Figure 3).

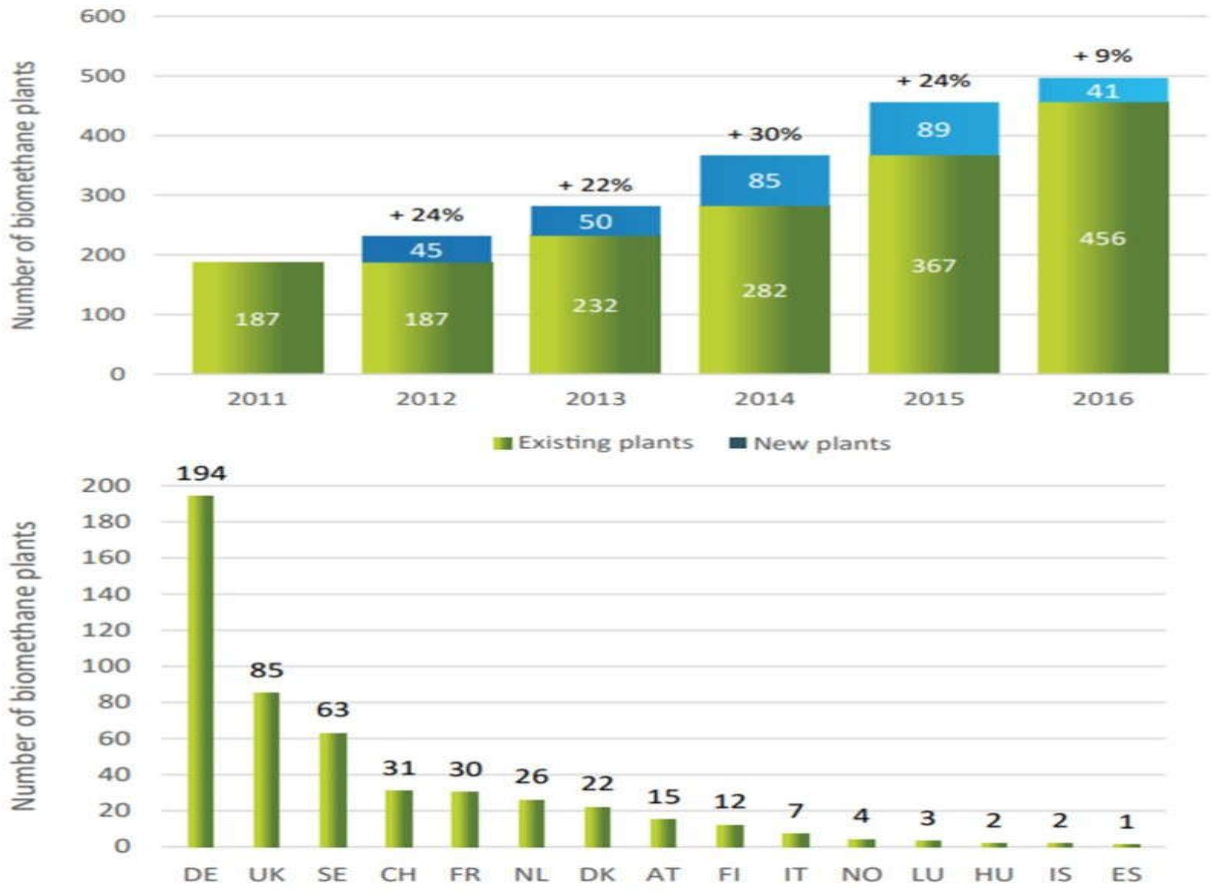


Figure 3. Number and distribution of biomethane plants in Europe (European Biogas Association, 2018).

1.3 Anaerobic Digestion

Anaerobic Digestion (AD) is a widespread process in natural ecosystems rich in organic matter and with low oxygen content, and also with low amounts of nitrate, sulphate, iron or manganese (Schink, 1997). These ecosystems include shallow freshwaters such as marshes, rice paddies,

submerged soils, but also human and animal intestinal tracts (large ruminant and non-ruminant herbivores and woodworms) and anthropogenic environments such as landfills and anaerobic digesters. For thermodynamic reasons, methanation is established when both oxygen and other thermodynamically more convenient electron acceptors are consumed (Liu e Whitman, 2008) (Table 1), and this is why it rarely occurs in brackish water, usually sulphate-rich environments (Izzo et al., 2014). Methanogenesis is the last of the expected reactions along the scale of decreasing redox potentials.

Table 1. Thermodynamic sequence for the reduction of inorganic compounds at pH 7 (Liu e Whitman, 2008).

Reaction	E_h (V)	ΔG^b
Reduction of O_2 **$O_2 + 4H^+ + 4e^- \leftrightarrow 2H_2O$**	0.812	-29.9
Reduction of NO_3^- **$2NO_3- + 6H^+ + 6e^- \leftrightarrow N_2 + 3H_2O$**	0.747	-28.4
Reduction of Mn^{4+} to Mn^{2+} **$MnO_2 + 4H^+ + 2e^- \leftrightarrow Mn_2^+ + 2H_2O$**	0.526	-23.3
Reduction of Fe^{3+} to Fe^{2+} **$Fe(OH)_3 + 3H^+ + e^- \leftrightarrow Fe^{2+} + 3H_2O$**	-0.047	-10.1
Reduction of SO_4^{2-} to H_2S **$SO_4^{2-} + 10H^+ + 8e^- \leftrightarrow H_2S + 4H_2O$**	-0.221	-5.9
Reduction of CO_2 to CH_4 **$CO_2 + 8H^+ + 8e^- \leftrightarrow CH_4 + 2H_2O$**	-0.244	-5.6

The AD process consists of a sequence of anaerobic biochemical reactions involved in the degradation of complex organic molecules such as polysaccharides, lipids and proteins, into CH_4 and it can be recognized in four main phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as shown in Figure 4. It is carried out by at least four specialized microbial consortia that operate in syntrophic relationships through a chain of reactions up to the final catabolites, that are mainly methane and carbon dioxide. These consortia are distinguished both by the substrates used and by the products of their catabolism; each is characteristic of a single AD phase, and the metabolites produced by one group is used as substrates by the next group.

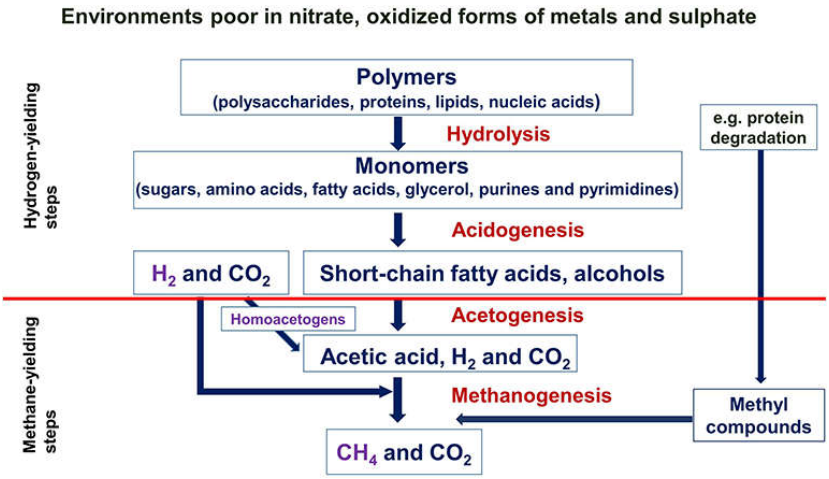


Figure 4. Scheme of anaerobic digestion process (Sikora et al., 2017).

A typical example of anaerobic digestion of a simple organic substrate is the degradation of glucose which occurs following the reaction:

$$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$$

$$CH_3COOH \rightarrow CH_4 + CO_2$$

For more complex substrates, other reaction products can be obtained; among those of greatest interest are nitrogen compounds, such as ammonia (NH_3), derived from protein degradation, or hydrogen sulfide (H_2S) produced by bacteria that perform the sulfate reduction.

The complexity and efficiency of the microbial community active in AD, both highlight the prevalence of cooperative type interactions evolved between different functional groups of microorganisms (Ghosh et al., 2016). These interactions, such as cooperation, mutualism, commensalism, all confer an advantage to at least one participant in the interactions and to the functional role of each group, i.e. in ecological terms we can say - each niche - contributes to the balance and stability of the AD process, as demonstrated by its wide spread in natural environments. The microorganisms involved in the AD process are therefore linked by close metabolic relationships that determine the formation of a trophic network in which different functional groups of microorganisms, called guilds, occupy specific ecological niches. In the different steps of the trophic chain, the products derived from the metabolism of one guild become the exclusive growth substrate of another (Kato et al., 2012). Within each guild (intra-guild), different microbial taxa may be replaced by others as a consequence of ecological conditions, but the functional metabolic role played and represented by each guild must be maintained. The guilds are therefore closely interconnected (inter-guild) both spatially and temporally, so that the entire

process is operationally considered as a *unicum* in which the production of CH_4 is only the last step. Stability and performance of the AD process depend on the balance of growth and the synchronization of the metabolic rate of the groups of microorganisms that cooperate in the realization of the trophic network (Demirel e Scherer, 2008), while it is believed that the lack of balance between guilds is often the primary cause of the instability of the whole process (Demirel et al., 2005).

The first three phases of AD are operated by microorganisms belonging to the Bacteria domain while CH_4 production is operated by methanogens from the Archaea domain. These two groups of microorganisms, belonging to domains of two such different evolutionary lineages have different physiology, different nutritional requirements, different growth kinetics and different sensitivity to environmental conditions (Pohland e Ghosh, 1971). At the metabolic interconnection between Bacteria and Archaea, a very special type of mutualism, termed syntrophy, has evolved.

1.3.1 Hydrolysis

In this first phase, a pool of different bacterial groups operate to degrade complex, particulate, or soluble organic substrates to form simpler compounds such as amino acids, fatty acids, and simple sugars.

The different hydrolytic bacteria act by colonizing particulate material by degrading it or by producing extracellular lytic enzymes that break down organic macromolecules into simpler, smaller monomers so that they can be used by fermentative acidogenic bacteria.

Hydrolysis of difficult-to-degrade polymers, such as cellulose or hemicellulose, often becomes the limiting step of the entire AD process; during solid waste digestion, approximately 50% of the organic material remains untreated due to lack by microorganisms of the enzymes necessary for its degradation (Ali Shah et al., 2014).

Previous studies showed that proteolytic bacteria acting during AD process are mainly represented by members of class *Clostridia*, which are also capable for amino acids degradation (Ramsay e Pullammanappallil, 2001). Moreover, both *Clostridia* and *Bacteroidetes* phyla, play a fundamental role for carbohydrates degradation, while *Proteobacteria*, are also involved in lipids metabolism (Xia et al., 2014; Yue et al., 2013).

1.3.2 Acidogenesis

Acidogenesis is the process in which metabolites coming from hydrolysis are further broken down into simpler organic molecules thanks to the action of a specific group of bacteria, which oxidize the organic substrates produced during hydrolysis to pyruvate, which is subsequently transformed into Volatile Fatty Acids (VFAs), lactic acid, alcohols, H_2 and CO_2 which are the starting substrates for the subsequent acetogenic phase. Otherwise, H_2, CO_2 and acetate may be directly used by archaea methanogens. However, VFAs and gaseous products accumulation may occur if the produced substrates are not efficiently utilized by microorganism of the next phase. In particular, the H_2 partial pressure has an important role in this process. High H_2 partial pressure leads to propionate and butyrate accumulation, while the low H_2 partial pressure enhances CO_2 and CH_4 production (Liu e Whitman, 2008).

Microorganisms involved in acidogenesis belong to phylum *Firmicutes, Bacteroidetes, Synergistetes*, such as *Anaerobaculum* (Hahnke et al., 2015; Stolze et al., 2015).

1.3.3 Acetogenesis

Acetogenesis is the third phase in the trophic chain of AD. Starting from the volatile fatty acids, lactic acid, and alcohols formed during the previous acidogenesis, acetogenic bacteria produce acetic acid, CO_2, and H_2. It is the only endergonic phase of AD under standard conditions, i.e. thermodynamically unfavourable (Shink , 1997).

The level of H_2 in solution is maintained at a low concentration thanks to the action of hydrogenotrophic methanogenic bacteria or other hydrogen utilizing bacteria whom live in syntropic association with acetogenic bacteria such as homoacetogenes. Consequently, the latter are in competition with hydrogenotrophic methanogens for using H_2 as substrate (Dinamarca et al., 2011).This syntrophic relation between hydrogen producers and hydrogen consumers is known as interspecies hydrogen transfer (IET).

The acetate produced can be converted into CH_4 either directly by acetoclastic methanogens or through a further metabolic pathway known as syntrophic acetate oxidation (SAO) in which acetic acid is oxidized to $H_2 + CO_2$. SAO pathway can be operated by bacterial groups belonging to *Thermotogaceae, Synthrophomonadaceae* and *Thermoanaerobacteriaceae* families (Hattori, 2008).

1.3.4 Homoacetogenesis

In anaerobic environments, H_2 can be used as an electron donor in several metabolic pathways to generate acetate, or other intermediates such as formate or methanol, according to the following reactions:

$$2HCO_3^- + 4\ H_2 \rightarrow \text{Acetate-} + 4\ H_2O$$

$$HCO_3^- + H_2 \rightarrow \text{Formate-} + 4\ H_2O$$

$$HCO_3^- + 3\ H_2 \rightarrow \text{Methanol} + 2\ H_2O$$

Although hydrogenotrophic methanogens often do not compete interspecifically with homoacetogenic bacteria for hydrogen utilization since the latter have a higher activation threshold,, several studies (Agneessens et al., 2018; Dinamarca e Bakke, 2012; Zhu et al., 2019) have shown that the autotrophic H_2 consumption by homoacetogenic bacteria can play a key role within the AD process.

In agreement with Schink (1997), homoacetogenic bacteria are a very versatile group of anaerobic bacteria. When necessary, they are capable of switching from the heterotrophic acetogenic metabolism, with acetate consumption and $H_2 + CO_2$ production, to the autotrophic homoacetogenic metabolism, with identical metabolic reactions but with reverse direction, i.e. H_2 and CO_2 consumption to produce acetate. High rates of hydrogen production promote the switch of this community toward the second direction, aimed to the H_2 depletion (Regueira et al., 2018; Saady, 2013).

1.3.5 Methanogenesis and interspecies electron transfer

The production of methane is the conclusion of the whole trophic chain of AD; in fact it is the unique stable and non-reactive compound among all the intermediates of the whole process and therefore it can be considered the real final product.

The formation of methane occurs through two main pathways, each operated by a different group of specialized microorganisms. The complete methanogenesis reaction that ca occurs during AD is shown in Table 2.

Table 2 Methanongenesis reactions (Demirel e Scherer, 2008)

1	Hydrogen $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
2	Acetate $CH_3COOH \rightarrow CH_4 + CO_2$
3	Formate $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$
4	Methanol $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$
5	Carbon monoxide $4CO + 2H_2O \rightarrow CH_4 + 3H_2CO_3$
6	Trimethylamine $4(CH_3)3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$
7	Dimethylamine $2(CH_3)2NH + 2H_2O \rightarrow 3\ CH_4 + CO_2 + 2NH_3$
8	Monomethylamine $4(CH_3)NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4\ NH_3$
9	Methyl mercaptans $2(CH_3)_2S + 3H_2O \rightarrow 3CH_4 + CO_2 + H_2S$
10	Metals $4Me^0 + 8H^+ + CO_2 \rightarrow 4Me^{++} + CH_4 + 2H_2O$

Usually, 70% of the CH_4 production occurs by the acetoclastic pathway, operated by acetotrophic methanogens by the "clivage" of the acetate into CH_4 and CO_2.

$$CH_3COOH \rightarrow CH_4 + CO_2$$

Methanosaeta and *Methanosarcina* both represent the dominant family in methanogenic members (Liu e Whitman, 2008) but only *Methanosarcinaceae* are capable of producing CH_4 by hydrogenotrophic and acetoclastic routes (Demirel e Scherer, 2008).

The second pathway for methane production is operated by hydrogenotrophic bacteria through the reduction of CO_2 using H_2 according to the following reaction:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$$

Hydrogenotrophic methanogens include five Orders: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, and Methanopyrales* (Zabranska e Pokorna, 2018). Among *Methanosarcinales*, the acetogenic members of *Methanosaet*a genus are able to convert acetate produced during the acidogenesis and the acetogenesis phases, to CH_4 and CO_2. In this way,

they contribute to maintain the pH stable, preventing the inhibition due to high acidity. Members of the genus *Methanosarcina* obtain energy from the reduction of CO_2 by using H_2 or CO as the electron donor. *Methanobacteriales, Methanococcales, Methanomicrobiales, and Methanopyrales* microorganisms obtain energy through the reduction of CO_2 to CH_4 by using H_2 and they are referred as CO_2-reducing obligate species (Ferry, 2010). They play a key role in maintaining H_2 partial pressure below critical concentrations (<10 Pa) by allowing acetogenesis from syntrophic oxidation of propionate and butyrate.

The syntrophic interaction that occurs between H_2-producing organisms (e.g., propionate-oxidizing bacteria) and H_2-consuming organisms (e.g., homacetogens and hydrogenotrophic methanogens) is termed by "interspecies electron transfer (IET)" and allows for the cooperative transformation of organic compounds into methane (Baek et al., 2018; Stams e Plugge, 2009). Therefore, the effect of exogenous hydrogen addition can directly boost hydrogenotrophic methanogenesis and achieve the desired conversion of CO_2 into extra methane production. Alternatively, also homoacetogens can be 'partial' consumers of the added H_2 (Agneessens et al., 2018).

1.3.6 Syntrophy: ecological and energetic aspects

Shink (1997) defined syntrophy as "a special case of symbiotic cooperation, evolved between two philogenetically distant types of microorganisms, which depend on each other for the degradation of a given substrate, typically for energetic reasons."

The term should be limited to cases where mutual dependence cannot be overcome simply by adding a substrate or any type of nutrient. The low energy available in methanogenic conversion, is the key aspect that has evolutionarily forced microorganisms into such efficient cooperation. .Indeed,, syntrophic bacteria, are unable to carry out the fatty acid oxidation to produce acetate, since they need to be associated with syntrophic partner that consumes H_2. The energetic of fermentation reactions of acids such as butyrate, propionate, etc., would provide a positive free energy change ($\Delta G°$) if the these reactions proceed under standard conditions (solutes:1M; gas:1 atm; T:25°C) (Table 3). In other words, energy would be consumed instead of released. On the other hand, the consumption of H_2 and the consequent reduction of its partial pressure, drastically affects the energy of the reaction, making it favourable, that is, with ΔG of negative values. This occurs when the concentrations of H_2 are maintained close to zero by H_2 consumer microorganisms. It is believed that in some syntrophic association, the transfer of H_2 takes place by direct conduction, by means of structures similar to electrical cables and therefore capable of conducting

electricity. Schink (2006) states that many of the fermentation reactions that occur during the AD process are endergonic under standard conditions, i.e. thermodynamically unfavourable, but they become exergonic, and therefore spontaneous without energy consumption, if implemented in sequence as if they were a single reaction. This can occur under the same anaerobic conditions founded in marine sediments and within anaerobic digesters.

Table 3. Variation in Gibbs free energy (ΔG0') under standard conditions during H_2 production reactions by oxidation of some VFAs produced in Anaerobic Digestion (Shink 1997).

Reaction	**$\Delta G^{0'}$ (kJ for mole)**
Acetate + H^+ + H_2O → 2 CO_2 + 4H_2	+94.9
Butyrate + 2H_2O → 2 Acetate + H^+ + H_2	+48.3
Propionate + 2H_2O → Acetate + CO_2 + 3H_2	+76
Valerate + CO_2 + H_2O -> 3 Acetate + 2H^+ + 2H_2	+ 25.2
Ethanol + H_2O → Acetate + H_2	+ 9.6

Methanogenesis is also an exergonic metabolic process but is poorly efficient: conversion of a hexose to CH_4 releases only 15% of the energy obtainable from aerobic degradation:

Aerobic respiration: $C_6H_{12}O_6$+ 6 O_2 ->6CO_2 + 6H_2O *($\Delta G°' = -2870$ kJ mole^{-1})*

Methanogenesis: $C_6H_{12}O_6$ -> 3CO_2 + 3 CH_4 *($\Delta G°' = -390$ kJ mole^{-1})*

Thus, the coevolution of syntrophic relationships supports the interspecies transfer of both H_2 and acetate between acetotrophic bacteria, which produce these compounds, and methanogenic Archaea, which use them: in this way, methanogens allow to keep the partial pressure of H_2 low ($<10^{-4}$ atm), allowing the acetogenesis reactions to become thermodynamically favourable, and thus allowing the thermodynamic stability of the whole AD process (Schink, 2006). The energy balances of these interactions are very complex, although the Gibbs free energy of exchange still remains very small. This highlights how through syntrophic relationship, Bacteriae and Archaea are able to survive by sharing very small amounts of energy, having by necessity co-evolved very efficient catabolic systems such that they can proliferate in thermodynamically unfavourable environments (Lane et al., 2013).

The need for such close energetic cooperation causes microorganisms involved in syntrophy to assume a well-structured spatial arrangement (Felchner-Zwirello et al., 2013; O'Flaherty et al., 2006). Indeed, the formation of cellular aggregates, mainly in floccules or granules, between guilds

of functionally different microorganisms facilitates metabolic exchanges and the transfer of electrons and H_2 (Ishii et al., 2006; Khemkhao et al., 2011; Shen et al., 2016). Several hypotheses have been formulated regarding the formation of these aggregates (Hulshoff Pol et al., 2004), but however the arrangement that appears to be most effective is the one defined as multi-layer, suggested by McIneney et al., 2008 (Figure 5).

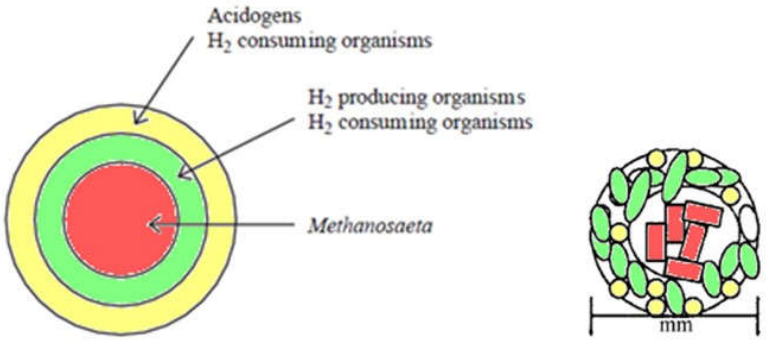


Figure 5. A) Structure of cellular aggregates consisting of different guilds of microorganisms active in AD (McInerney et al., 2008); B) Representation of granule structure according to (Khemkhao et al., 2011): central core consisting of methanogenic Archaea (in red) surrounded by syntrophic acetogenic bacteria (in green) and externally by acidogenic bacteria (in yellow). Aggregate sizes can reach the order of millimeters.

1.4 Biogas upgrading technologies

1.4.1 Physical and chemical technologies

Technologies for biogas upgrading to biomethane currently available commercially are based on the removal of CO_2 present in biogas through chemical-physical processes, i.e.water scrubbing, organic solvents pressure swing adsorption (PSA) and membrane separation (Muñoz et al., 2015). Moreover, there are other technologies based on cryogenic separation or chemical hydrogenation, which are still under development (Angelidaki et al., 2018).

If on the one hand the methane recovery from physicochemical processes can reach over 96% on the other hand high pressures, high operating temperatures or addition of chemicals are required to ensure an efficient methanation with further energetic and environmental repercussions. (Angelidaki et al., 2018; Sun et al., 2015).Moreover, some biogas components released from biogas upgrading are associated with GHG, especially CO_2. The direct impacts of excessive CO_2 emission are global warming, ocean acidification, and carbon fertilization (Adnan et al., 2019).The released CO_2 needs to be disposed. For these reasons, alternative upgrade technologies are desirable.

1.4.2 Biological upgrading

An attractive and cost effective method to increase the CH_4 content in biogas is based on the biological reduction of CO_2 in the biogas to CH_4. It is operated by a specific group of microorganism able to convert H_2 and CO_2 into CH_4 by following reaction (Eq.1):

$$(Eq.1) \quad 4\ H_2 + CO_2 = CH_4 + 2\ H_2O \qquad \Delta G^0 = -130\ KJ\ mol^{-1}$$

The advantages of this method are many, milder operating conditions (i.e. atmospheric pressure and moderate temperatures), no use of chemicals. Moreover biological fixation of CO_2 is a sustainable solution to reduce CO_2 content in biogas due to its nature which is environmentally-friendly and eliminates the step of captured CO_2 disposal. These conditions could significantly reduce investment and operational costs compared to currently available commercial technologies (Agneessens et al., 2018; Fang et al., 2020).

1.4.3 Power to Gas

In order to make biological upgrading a sustainable process, the H_2 required in the reaction (Eq. 1) should be derived from a renewable source. The required H_2 can be produced by biomass gasification, biomethane reforming, biological H_2 production, or through water electrolysis (Luo et al., 2012; Turner et al., 2008).

Currently, water electrolysis using renewable energy sources as solar and wind power is considered the unique environmentally friendly technology applicable on a large scale to obtain H_2 for bioconversion of CO_2 to CH_4. Three different electrolysis technologies are of interest for renewable H_2 production: alkaline electrolysis (AEL), polymer electrolyte membranes (PEM), and solid oxide electrolysis (SOEC) (Gotz et al., 2016).

On the other hand, given the fluctuating and intermittent nature of solar and wind energies and the consequent mismatches between supply and electrical demand, H_2 generation from these energy sources, allow the long-term electricity storage (Bailera et al., 2017).

Therefore, coupling biogas and CO_2 production from anaerobic digestion of organic matter with the exploitation of H_2 that is generated due to excess renewable energy is an effective method for bioenergy production by creating a unique synergy of renewable energy sources called Power-to-Gas (P2G) (Götz et al., 2016; Kougias et al., 2017) converting electricity into a chemical energy

carrier, which can be easily stored and transported in the existing natural gas infrastructure (Figure 6).

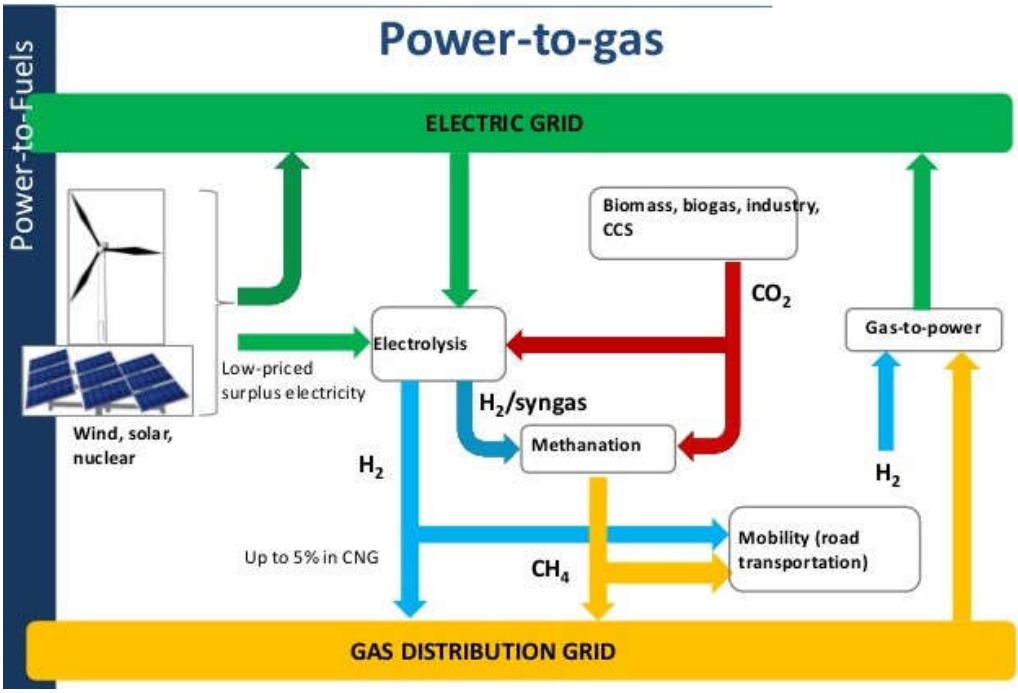


Figure 6. Power-to-Gas conceptual scheme

1.5 Configurations of biological upgrading

Two main applications of biological upgrading of biogas have been studied: 1) the *in situ* upgrading in which H_2 is injected directly into the same digester where AD takes place using internally CO_2 and 2) *the ex situ* upgrading, where H_2 and exsternal source of CO_2 are supplied in a separate reactor containing an enriched hydrogenotrophic methanogens culture (Bassani et al., 2017; Luo e Angelidaki, 2013a; Zhang et al., 2020). Both biological upgrading technology are still under developing.

Briefly, the *ex situ* upgrading has different advantages when compared to the *in situ* upgrading:

• it does not affect the stability of the anaerobic digestion process since only the hydrogenotrophic methanogenesis step takes place;

• other external sources of residual CO_2 or CO can be fed to the upgrade unit, besides to CO_2 from an conventional AD process, making the overall process more flexible;

• The process is independent of the properties of the substrate used in the AD process.

Since the aim of this PhD thesis is the development of biomethanation strategies to modify the full-scale biogas plant reducing the initial investment cost and the plant operation complexity, the focus of the following paragraphs will be only on the *in situ* biological upgrading,

1.5.1 *In situ* Biological upgrading

In situ biological upgrading exploits the action of native hydrogenotrophic methanogenic archaea to convert the CO_2 produced in the digester (Figure 7).

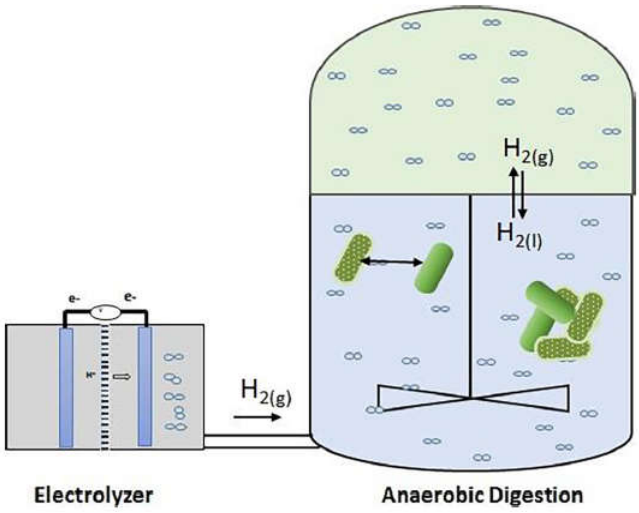


Figure 7. *In situ* biological biogas upgrading configuration's scheme (Aryal et al., 2018).

The H_2 diffusion in the liquid phase is the limiting factor of the process since the low H_2 solubility (Henry's constant KH = 7.40 x 10^{-9} mol L^{-1} Pa^{-1}) make it not bioavailable for the microbial community. In order to overcome this limit, several *in situ* strategies have been proposed, mainly introducing different H_2 injection devices. In small-scale reactor (working volume ≤ 3.5 L), the H_2 was introduced by column or ceramic diffusers (Luo et al., 2012), hollow fiber membrane (HFM) (Luo and Angelidaki, 2013) and headspace injection (Agneessens et al., 2017), all using different stirring speeds (65-1000 rpm) or biogas recirculation verifying a better utilization of hydrogen whose coupling with carbon dioxide leads to improved conversion to methane. (Alfaro et al., 2019; Bassani et al., 2017)

Moreover, injection of exogenous hydrogen into the digester, can lead to disadvantages to the anaerobic digestion process, such as increased pH, due to CO_2 removal, and process inhibition due to the higher partial pressure of H_2.

Regarding pH increase, it is known how the maintenance of pH levels in the optimal range for AD (6-8) is regulated by the concentration of H^+ according to the following equilibrium equation:

$$[H^+] = K1\frac{[H_2CO_2]}{[HCO_3^-]}$$

Where:

$[H_2CO_2]$ is the concentration of carbonic acid which is in equilibrium with the CO_2 content in the digester headspace; $[HCO_3^-]$ is the bicarbonate concentration in the liquid phase; K1 is the ionization constant for carbonic acid.

The pH increase is attributed to the removal of bicarbonate which is a key buffer in many biological processes, including AD. CO_2 in liquid phase in the form of bicarbonate is dissociated into H^+ and HCO_3^-. Therefore, the use of CO_2 by hydrogenotrophic methanogens will lead to a decrease in H^+ ions thus causing an increase in pH.

In literature, this result has already been demonstrated for the *in situ* upgrading of biogas with pH values above 8.5 which resulted in a inhibition of methanogenesis (Bassani et al., 2015; Luo e Angelidaki, 2013a). In order to control the pH into suitable range for AD process two ways were tested: co-digestion with an acidic waste such as cheese whey (Luo e Angelidaki, 2013b; Treu et al., 2019) or instrumental pH control, but in this case , significant alkalinity consumption (-73%) was observed at the final value below 1000 mg L^{-1} due to the addition of HCl (Wang et al., 2013).

Another important issue that has to be considered during the *in situ* biogas upgrading process is related with the oxidation of Volatile Fatty Acids (VFA) and alcohols which is only thermodynamically feasible in cases that H_2 concentration is very low (<10 Pa) On contrary, high H_2 levels (> 10 Pa) inhibit the anaerobic digestion and promote the accumulation of reaction intermediates such as butyrate, lactate, propionate caused by the inhibition of acetogenic bacteria (Liu e Whitman, 2008).

An high H_2 partial pressure can also promote the establishment of homoacetogenesis, an alternative metabolic pathway antagonist to hydrogenotrophic methanogenesis for the use of H_2 according to the following equation:

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G^0 = -104.6\, kJ\ mol^{-1}$$

The significant presence of homoacetogenic bacteria activity explains a possible decrease in the rate of methane production (Agneessens et al., 2018).

Table 4 summarizes the *in situ* setup studies.

Table 4. Summary of literature studies of the *in situ* technology integration of data from Angelidaki et al., 2018.

Organic Substrate	Reactor type	OLR (gVS/ L/d)	T (°C)	pH	HRT(d)	H_2(L/Lr/d)	H_2/CO_2	H2 conversion (%)	CO_2 removal (%)	CH_4 (%)	Ref.
potato starch	UASB	2.79	55	8.38	7	3.5	04:01	67	76	82	(Bassani et al., 2016)
cattle manure	CSTR	1.85	55	8.3	14	1.8	04:01	>90		65	(Luo et al., 2012)
cattle manure and cheese whey	CSTR	1.66	55	7.7 7,9	15	1.5-1.7	04:01		85	75	(Luo and Angelidaki 2013a)
sewage sludge	CSTR	0.77	38	7.89 - 8,43	20	0.3-1.7	2:1-10:1	58-99	43.3-100	76.8- 100	(Agneessens et al., 2017)
cattle manure and cheese whey	CSTR	1.66	55	7.61 - 8,31	15	0.93- 1,76	04:01		53-91	78.4- 96.1	(Luo and Angelidaki, 2013b)
sewage sludge	CSTR	2	37	8.0	10	0.6-1,32	3.27:1 -5.4:1	96	99	98.9	(Wang et al., 2013)

Other important parameters to take into consideration are the amount of H_2 to inject into the reactor in relation to the CO_2 produced by AD and the H_2 conversion rate achieved.

Among the previous studies, only two reported almost pure methane at the gas outlet (98.9% and 100%). Wang et al., (2013) they did not use pure H_2 but synthetic coke oven gas (SCOG) composed of H_2 and CO, taking into account the CO added and the amount of CO_2 derived from the biogas produced *in situ*. An overall H_2 / CO_2 ratio ranging from 3.3: 1 to 5.4: 1 was adopted and a methane content of 100% was obtained. Agneessens et al. (2017) tested a semi-batch condition and adopting a pulse addition of H_2 / CO_2 ratio from 2: 1 to 10: 1 ratio to daily produced CO_2. At H_2 injection rates exceeding 6: 1 to CO_2 ratio a complete upgrading of the CH_4 fraction of the produced biogas from 59. 4 ± 9.0 % to 100% was achieved. As long as pH remained below 8.18. On the other hand, at higher pH values, only 58% of the H_2 consumed was transformed into methane. The remaining fraction of H_2 injected was probably used by homoacetogenic bacteria and converted into acetate which increased 10-fold compared to the control reactor.

Furthermore, this study suggests close monitoring of H_2 consumption rate during H_2 injections to increase methane production and control of volatile fatty acid concentrations. Thus, although an indication that working above the stoichiometric value might allow for higher methane content, further research on the relevance of the H_2/ CO_2 ratio remains to be addressed. Current and previous studies have operated at or above stoichiometric ratio, but always considering CO_2 only in the headspace.

Another parameter poorly studied to date is the rate of recirculation of the biogas inside the reactor. This parameter is very important because it greatly influences the bioavailability of H_2 for the bacterial community, and therefore the possibility of increasing the fraction of H_2 converted into CH_4 (Alfaro et al., 2019).

1.6 References

Agneessens, L.M., Ottosen, L.D.M., Andersen, M., Berg Olesen, C., Feilberg, A., Kofoed, M.V.W., 2018. Parameters affecting acetate concentrations during *in-situ* biological hydrogen methanation. Bioresour. Technol. 258, 33–40.

Agneessens, L.M., Ottosen, L.D.M., Voigt, N.V., Nielsen, J.L., de Jonge, N., Fischer, C.H., Kofoed, M.V.W., 2017. *In-situ* biogas upgrading with pulse H_2 additions: The relevance of methanogen adaption and inorganic carbon level. Bioresour. Technol. 233, 256–263.

Ahmad, T., Aadil, R.M., Ahmed, H., Rahman, U. ur, Soares, B.C.V., Souza, S.L.Q., Pimentel, T.C., Scudino, H., Guimarães, J.T., Esmerino, E.A., Freitas, M.Q., Almada, R.B., Vendramel, S.M.R., Silva, M.C., Cruz, A.G., 2019. Treatment and utilization of dairy industrial waste: A review. Trends Food Sci. Technol. 88, 361–372.

Alfaro, N., Fdz-Polanco, M., Fdz-Polanco, F., Díaz, I., 2019. H_2 addition through a submerged membrane for *in-situ* biogas upgrading in the anaerobic digestion of sewage sludge. Bioresour. Technol. 280, 1–8.

Ali Shah, F., Mahmood, Q., Maroof Shah, M., Pervez, A., Ahmad Asad, S., 2014. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. Sci. World J. 2.

Amann, R., Binder, B., Olson, R., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Envir. Microbiol. 56, 1919–1925.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-olignucleotide probing of whole cells for determinitive, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. Biotechnol. Adv. 36, 452–466.

Angelidaki, I., Xie, L., Luo, G., Zhang, Y., Oechsner, H., Lemmer, A., Munoz, R., Kougias, P.G., 2019. Biogas upgrading: Current and emerging technologies, Biomass, Biofuels, Biochemicals: Biofuels: Alternative Feedstocks and Conversion Processes for the Production of Liquid and Gaseous Biofuels.

Aryal, N., Kvist, T., Ammam, F., Pant, D., Ottosen, L.D.M., 2018. An overview of microbial biogas enrichment. Bioresour. Technol. 264, 359–369.

Asunis, F., Gioannis, G. De, Dessì, P., Isipato, M., Lens, P.N.L., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2020. The dairy biorefinery : Integrating treatment processes for cheese whey valorisation. J. Environ. Manage. 276, 111240.

Awe, O.W., Zhao, Y., Nzihou, A., Minh, D.P., Lyczko, N., 2017. A Review of Biogas Utilisation, Purification and Upgrading Technologies. Waste and Biomass Valorization 8, 267–283.

Baek, G., Kim, Jaai, Kim, Jinsu, Lee, C., 2018. Role and potential of direct interspecies electron transfer in anaerobic digestion. Energies 11, 107–125.

Barra Caracciolo, A., Grenni, P., Cupo, C., 2005. *In situ* analysis of native microbial communities in complex samples with high particulate loads. FEMS Microbiol. 253, 55–58.

Bassani, I., Kougias, P.G., Angelidaki, I., 2016. *In-situ* biogas upgrading in thermophilic granular UASB reactor: key factors affecting the hydrogen mass transfer rate. Bioresour. Technol. 221, 485–491.

Bassani, I., Kougias, P.G., Treu, L., Angelidaki, I., 2015. Biogas Upgrading via Hydrogenotrophic Methanogenesis in Two-Stage Continuous Stirred Tank Reactors at Mesophilic and Thermophilic Conditions. Environ. Sci. Technol. 49, 12585–12593.

Bassani, I., Kougias, P.G., Treu, L., Porté, H., Campanaro, S., Angelidaki, I., 2017. Optimization of hydrogen dispersion in thermophilic up-flow reactors for *ex situ* biogas upgrading. Bioresour. Technol. 234, 310–319.

Braga Nan, L., Trably, E., Santa-Catalina, G., Bernet, N., Delgenès, J.P., Escudié, R., 2020. Biomethanation processes: New insights on the effect of a high H_2 partial pressure on microbial communities. Biotechnol. Biofuels 13, 1–18.

British Petroleum Report, 2018. BP Statistical Review of World Energy 67th edition.

Burkhardt, M., Busch, G., 2013. Methanation of hydrogen and carbon dioxide. Appl. Energy 111, 74–79.

Campanaro, S., Treu, L., Kougias, P.G., De Francisci, D., Valle, G., Angelidaki, I., 2016. Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnol. Biofuels 9, 26.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat. Publ. Gr. 7, 335–336.

Carvalho, F., Prazeres, A.R., Rivas, J., 2013. Cheese whey wastewater: Characterization and treatment. Sci. Total Environ. 445–446, 385–396.

De Francisci, D., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2015. Microbial diversity and dynamicity of biogas reactors due to radical changes of feedstock composition. Bioresour. Technol. 176, 56–64.

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Rev. Environ. Sci. Biotechnol. 7, 173–190.

Demirel, B., Yenigun, O., Onay, T.T., 2005. Anaerobic treatment of dairy wastewaters: A review. Process Biochem. 40, 2583–2595.

Diamantis, V.I., Kapagiannidis, A.G., Ntougias, S., Tataki, V., Melidis, P., Aivasidis, A., 2014. Two-stage CSTR-UASB digestion enables superior and alkali addition-free cheese whey treatment. Biochem. Eng. J. 84, 45–52.

Díaz, I., Pérez, C., Alfaro, N., Fdz-Polanco, F., 2015. A feasibility study on the bioconversion of CO_2 and H_2 to biomethane by gas sparging through polymeric membranes. Bioresour. Technol. 185, 246–253.

Dinamarca, C., Bakke, R., 2012. Simultaneous hydrogen production and consumption in Anaerobic mixed culture fermentation. Int. J. Energy Environ. 3, 323–332.

Dinamarca, C., Gañán, M., Liu, J., Bakke, R., 2011. H_2 consumption by anaerobic non-methanogenic mixed cultures. Water Sci. Technol. 63, 1582–1589.

European Biogas Association, 2018. EBA Statistical Report 2018. Annu. Rep. 68.

Faisal, S., Salama, E.S., Hassan, S.H.A., Jeon, B.H., Li, X., 2020. Biomethane enhancement via plastic carriers in anaerobic co-digestion of agricultural wastes. Biomass Convers. Biorefinery.

Felchner-Zwirello, M., Winter, J., Gallert, C., 2013. Interspecies distances between propionic acid degraders and methanogens in syntrophic consortia for optimal hydrogen transfer. Appl. Microbiol. Biotechnol. 97, 9193–9205.

Fernández, C., Cuetos, M.J., Martínez, E.J., Gómez, X., 2015. Thermophilic anaerobic digestion of cheese whey: Coupling H 2 and CH 4 production. Biomass and Bioenergy 81, 55–62.

Ferraro, A., Massini, G., Mazzurco Miritana, V., Rosa, S., Signorini, A., Fabbricino, M., 2020. A novel enrichment approach for anaerobic digestion of lignocellulosic biomass: Process performance enhancement through an inoculum habitat selection. Bioresour. Technol. 313, 123703–123712.

Ferry, J.G., 2010. The chemical biology of methanogenesis. Planet. Space Sci. 58, 1775–1783.

Fontana, A., Campanaro, S., Treu, L., Kougias, P.G., Cappa, F., Morelli, L., Angelidaki, I., 2018a. Performance and genome-centric metagenomics of thermophilic single and two-stage anaerobic digesters treating cheese wastes. Water Res. 134, 181–191.

Fontana, A., Kougias, P.G., Treu, L., Kovalovszki, A., Valle, G., Cappa, F., Morelli, L., Angelidaki, I., Campanaro, S., 2018b. Microbial activity response to hydrogen injection in thermophilic anaerobic digesters revealed by genome-centric metatranscriptomics. Microbiome 6, 1–14.

Gagliano, M.C., Braguglia, C.M., Petruccioli, M., Rossetti, S., 2015. Ecology and biotechnological potential of the thermophilic fermentative Coprothermobacter spp. FEMS Microbiol. Ecol. 91, 1–12.

Ghaib, K., Ben-Fares, F.Z., 2018. Power-to-Methane: A state-of-the-art review. Renew. Sustain. Energy Rev. 81, 433–446.

Ghosh, S., Chowdhury, R., Bhattacharya, P., 2016. Mixed consortia in bioprocesses: role of microbial interactions. Appl. Microbiol. Biotechnol. 100, 4283–4295.

Goli, A., Shamiri, A., Khosroyar, S., Talaiekhozani, A., Sanaye, R., Azizi, K., 2019. A review on different aerobic and anaerobic treatment methods in dairy industry wastewater. J. Environ. Treat. Tech. 7, 113–141.

Gorrasi, S., Izzo, G., Massini, G., Signorini, A., Barghini, P., Fenice, M., 2014. From polluting seafood wastes to energy. production of hydrogen and methane from raw chitin material by a two-phase process. J. Environ. Prot. Ecol. 15, 526–536.

Götz, M., Lefebvre, J., Mörs, F., McDaniel Koch, A., Graf, F., Bajohr, S., Reimert, R., Kolb, T., 2016. Renewable Power-to-Gas: A technological and economic review. Renew. Energy 85, 1371–1390.

Guebitz, G.M., Bauer, A., Bochmann, G., Gronauer, A., Weiss, S., 2015. Biogas Science and Technology, Editors: Guebitz, G.M., Bauer, A., Bochmann, G., Grounauer, A., Weiss, S.

Gujer, W., Zehnder, A.J.B., 1983. Conversion processes in anaerobic digestion. Water Sci. Technol. 15, 127–167.

Hattori, S., 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. 23, 118–127.

Ho, D., Jensen, P., Batstone, D., 2014. Effects of temperature and hydraulic retention time on acetotrophic pathways and performance in high-rate sludge digestion. Environ. Sci. Technol. 48, 6468–6476.

Hulshoff Pol, L.W., De Castro Lopes, S.I., Lettinga, G., Lens, P.N.L., 2004. Anaerobic sludge granulation. Water Res. 38, 1376–1389.

Ishii, S., Kosaka, T., Hotta, Y., Watanabe, K., 2006. Simulating the contribution of coaggregation to interspecies hydrogen fluxes in syntrophic methanogenic consortia. Appl. Environ. Microbiol. 72, 5093–5096.

Izzo, G., Rosa, S., Massing, G., Patriarca, C., Fenice, M., Fiocchetti, F., Marone, A., Varrone, C., Signorini, A., 2014. From hypertrophic lagoons to bioenergy production. J. Environ. Prot. Ecol. 15, 537–546.

Jabari, L., Gannoun, H., Cayol, J.L., Hamdi, M., Fauque, G., Ollivier, B., Fardeau, M.L., 2012. Characterization of defluviitalea saccharophila gen. nov., sp. nov., a thermophilic bacterium isolated from an upflow anaerobic filter treating abattoir wastewaters, and proposal of defluviitaleaceae fam. nov. Int. J. Syst. Evol. Microbiol. 62, 550–555.

Kapoor, R., Ghosh, P., Kumar, M., Vijay, V.K., 2019. Evaluation of biogas upgrading technologies and future perspectives: a review. Environ. Sci. Pollut. Res. 26, 11631–11661.

Karadag, D., Köroılu, O.E., Ozkaya, B., Cakmakci, M., 2015. A review on anaerobic biofilm reactors for the treatment of dairy industry wastewater. Process Biochem. 50, 262–271.

Kato, S., Hashimoto, K., Watanabe, K., 2012. Microbial interspecies electron transfer via electric currents through conductive minerals. Proc. Natl. Acad. Sci. U. S. A. 109, 10042–10046.

Khemkhao, M., Nuntakumjorn, B., Techkarnjanaruk, S., Phalakornkule, C., 2011. Effect of chitosan on UASB treating POME during a transition from mesophilic to thermophilic conditions. Bioresour. Technol. 102, 4678–4681.

Koller, M., Gasser, I., Schmid, F., Berg, G., 2011. Linking ecology with economy: Insights into polyhydroxyalkanoate-producing microorganisms. Eng. Life Sci. 11, 222–237.

Kougias, P.G., Angelidaki, I., 2018. Biogas and its opportunities—A review. Front. Environ. Sci. Eng. 12, 1–12.

Kougias, P.G., Treu, L., Benavente, D.P., Boe, K., Campanaro, S., Angelidaki, I., 2017. *Ex-situ* biogas upgrading and enhancement in different reactor systems. Bioresour. Technol. 225, 429–437.

Krakat, N., Schmidt, S., Scherer, P., 2011. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. Bioresour. Technol. 102, 5692–5701.

Lane, N., Martin, W.F., Raven, J.A., Allen, J.F., 2013. Energy, genes and evolution: Introduction to an evolutionary synthesis. Philos. Trans. R. Soc. B Biol. Sci. 368, 1–5.

Lecker, B., Illi, L., Lemmer, A., Oechsner, H., 2017. Biological hydrogen methanation – A review. Bioresour. Technol. 245, 1220–1228.

Lembo, G., Massini, G., Mazzurco Miritana, V., Fenice, M., Felici, C., Liberatore, R., Signorini, A., 2016. Anaerobic digestion of ricotta cheese whey : effect of phase separation on methane production and microbial community structure, in: European Biomass Conference and Exhibition Proceedings, 2016 (24thEUBCE). ETA-Florence Renewable Energies, pagg. 599–607.

Lembo, G., Rosa, S., Miritana, V.M., Marone, A., Massini, G., Fenice, M., Signorini, A., 2021. Thermophilic anaerobic digestion of second cheese whey: Microbial community response to H_2 addition in a partially immobilized anaerobic hybrid reactor. Processes 9, 1–16.

Li, D., Ran, Y., Chen, L., Cao, Q., Li, Z., Liu, X., 2018. Instability diagnosis and syntrophic acetate oxidation during thermophilic digestion of vegetable waste. Water Res. 139, 263–271.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171–189.

Luo, G., Angelidaki, I., 2013a. Hollow fiber membrane based H_2 diffusion for efficient *in situ* biogas upgrading in an anaerobic reactor. Appl. Microbiol. Biotechnol. 97, 3739–3744.

Luo, G., Angelidaki, I., 2013b. Co-digestion of manure and whey for *in situ* biogas upgrading by the addition of H_2: Process performance and microbial insights. Appl. Microbiol. Biotechnol. 97, 1373–1381.

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., Angelidaki, I., 2012. Simultaneous hydrogen utilization and *in situ* biogas upgrading in an anaerobic reactor. Biotechnol. Bioeng. 109, 1088–1094.

Maegaard, K., Garcia-Robledo, E., Kofoed, M.V.W., Agneessens, L.M., de Jonge, N., Nielsen, J.L., Ottosen, L.D.M., Nielsen, L.P., Revsbech, N.P., 2019. Biogas upgrading with hydrogenotrophic methanogenic biofilms. Bioresour. Technol. 287, 121422.

Mainardis, M., Flaibani, S., Trigatti, M., Goi, D., 2019. Techno-economic feasibility of anaerobic digestion of cheese whey in small Italian dairies and effect of ultrasound pre-treatment on methane yield. J. Environ. Manage. 246, 557–563.

Martin, M.R., Fornero, J.J., Stark, R., Mets, L., Angenent, L.T., 2013. A single-culture bioprocess of methanothermobacter thermautotrophicus to upgrade digester biogas by CO_2-to-CH_4 conversion with H_2. Archaea 2013, 157529.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J.M., Schink, B., Rohlin, L., Gunsalus, R.P., 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Ann. N. Y. Acad. Sci. 1125, 58–72.

Mulat, D.G., Mosbæk, F., Ward, A.J., Polag, D., Greule, M., Keppler, F., Nielsen, J.L., Feilberg, A., 2017. Exogenous addition of H_2 for an *in situ* biogas upgrading through biological reduction of carbon dioxide into methane. Waste Manag. 68, 146–156.

Muñoz, R., Meier, L., Diaz, I., Jeison, D., 2015. A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Rev. Environ. Sci. Biotechnol. 14, 727–759.

Nakasaki, K., Nguyen, K.K., Ballesteros, F.C., Maekawa, T., Koyama, M., 2020. Characterizing the microbial community involved in anaerobic digestion of lipid-rich wastewater to produce methane gas. Anaerobe 61, 102082.

Narayanan, C.M., Narayan, V., 2019. Biological wastewater treatment and bioreactor design: A review. Sustain. Environ. Res. 1, 1–17.

Narihiro, T., Terada, T., Ohashi, A., Kamagata, Y., Nakamura, K., Sekiguchi, Y., 2012. Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment systems by sequence-specific rRNA cleavage method. Water Res. 46, 2167–2175.

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739.

O'Flaherty, V., Collins, G., Mahony, T., 2006. The microbiology and biochemistry of anaerobic bioreactors with relevance to domestic sewage treatment. Rev. Environ. Sci. Biotechnol. 5, 39–55.

Patterson, T., Esteves, S., Dinsdale, R., Guwy, A., Maddy, J., 2013. Life cycle assessment of biohydrogen and biomethane production and utilisation as a vehicle fuel. Bioresour. Technol. 131, 235–245.

Pavan, M.E., Pavan, E.E., Glaeser, S.P., Etchebehere, C., Kämpfer, P., Pettinari, M.J., López, N.I., 2018. Proposal for a new classification of a deep branching bacterial phylogenetic lineage: Transfer of Coprothermobacter proteolyticus and Coprothermobacter platensis to Coprothermobacteraceae fam. nov., within Coprothermobacterales ord. nov., Coprothermobacte. Int. J. Syst. Evol. Microbiol. 68, 1627–1632.

Pervin, H.M., Dennis, P.G., Lim, H.J., Tyson, G.W., Batstone, D.J., Bond, P.L., 2013. Drivers of microbial community composition in mesophilic and thermophilic temperature-phased anaerobic digestion pre-treatment reactors. Water Res. 47, 7098–7108.

Pohland, F.G., Ghosh, S., 1971. Developments in anaerobic stabilization of organic wastes - the two-phase concept. Environ. Lett. 1, 255–266.

Porté, H., Kougias, P.G., Alfaro, N., Treu, L., Campanaro, S., Angelidaki, I., 2019. Process performance and microbial community structure in thermophilic trickling biofilter reactors for biogas upgrading. Sci. Total Environ. 655, 529–538.

R.I. Amann, K.H. Ludwig, K.H.S., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Mol. Biol. Rev. 24, 143–169.

Rachbauer, L., Voitl, G., Bochmann, G., Fuchs, W., 2016. Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. Appl. Energy 180, 483–490.

Rajendran, K., Mahapatra, D., Venkatraman, A.V., Muthuswamy, S., Pugazhendhi, A., 2020. Advancing anaerobic digestion through two-stage processes: Current developments and future trends. Renew. Sustain. Energy Rev. 123, 109746.

Ramasamy, E.., Abbassi, S.., 2000. Energy recovery from dairy waste-waters: impacts of biofilm support systems on anaerobic CST reactors. Appl. Energy 65, 91–98.

Ramsay, I.R., Pullammanappallil, P.C., 2001. Protein degradation during anaerobic wastewater treatment: Derivation of stoichiometry. Biodegradation 12, 247–257.

Regueira, A., González-Cabaleiro, R., Ofiţeru, I.D., Rodríguez, J., Lema, J.M., 2018. Electron bifurcation mechanism and homoacetogenesis explain products yields in mixed culture anaerobic fermentations. Water Res. 141, 349–356.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3, 700–714.

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy 38, 13172–13191.

Sarker, S., Lamb, J.J., Hjelme, D.R., Lien, K.M., 2018. Overview of recent progress towards *in-situ* biogas upgradation techniques. Fuel 226, 686–697.

Schink, B., 2006. Syntrophic associations in methanogenic degradation. Prog. Mol. Subcell. Biol. 41, 1–19.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61, 262–280.

Shen, L., Zhao, Q., Wu, X., Li, X., Li, Q., Wang, Y., 2016. Interspecies electron transfer in syntrophic methanogenic consortia: From cultures to bioreactors. Renew. Sustain. Energy Rev. 54, 1358–1367.

Stams, A.J.M., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7, 568–577.

Sun, Q., Li, H., Yan, J., Liu, L., Yu, Z., Yu, X., 2015. Selection of appropriate biogas upgrading technology-a review of biogas cleaning, upgrading and utilisation. Renew. Sustain. Energy Rev. 51, 521–532.

Tandishabo, K., Nakamura, K., Umetsu, K., Takamizawa, K., 2012. Distribution and role of Coprothermobacter spp. in anaerobic digesters. J. Biosci. Bioeng. 114, 518–520.

Treu, L., Tsapekos, P., Peprah, M., Campanaro, S., Giacomini, A., Corich, V., Kougias, P.G., Angelidaki, I., 2019. Microbial profiling during anaerobic digestion of cheese whey in reactors operated at different conditions. Bioresour. Technol. 275, 375–385.

Turner, J., Sverdrup, G., Mann, M.K., Maness, P.C., Kroposki, B., Ghirardi, M., Evans, R.J., Blake, D., 2008. Renewable hydrogen production. Int. J. Energy Res. 32, 379–407.

Vasmara, C., Marchetti, R., 2017. Initial pH influences in-batch hydrogen production from scotta permeate. Int. J. Hydrogen Energy 42, 14400–14408.

Wang, W., Xie, L., Luo, G., Zhou, Q., Angelidaki, I., 2013. Performance and microbial community analysis of the anaerobic reactor with coke oven gas biomethanation and *in situ* biogas upgrading. Bioresour. Technol. 146, 234–239.

Xia, A., Cheng, J., Murphy, J.D., 2016. Innovation in biological production and upgrading of methane and hydrogen for use as gaseous transport biofuel. Biotechnol. Adv. 34, 451–472.

Xia, Y., Wang, Y., Fang, H.H.P., Jin, T., Zhong, H., Zhang, T., 2014. Thermophilic microbial cellulose decomposition and methanogenesis pathways recharacterized by metatranscriptomic and metagenomic analysis. Sci. Rep. 4, 1–9.

Yang, K., Yu, Y., Hwang, S., 2003. Selective optimization in thermophilic acidogenesis of cheese-whey wastewater to acetic and butyric acids : partial acidification and methanation 37, 2467–2477.

Yue, Z., Chen, R., Yang, F., MacLellan, J., Marsh, T., Liu, Y., Liao, W., 2013. Effects of dairy manure and corn stover co-digestion on anaerobic microbes and corresponding digestion performance. Bioresour. Technol. 128, 65–71.

Zabranska, J., Pokorna, D., 2018. Bioconversion of carbon dioxide to methane using hydrogen and hydrogenotrophic methanogens. Biotechnol. Adv. 36, 707–720.

Zhang, L., Kuroki, A., Tong, Y.W., 2020. A Mini-Review on *In situ* Biogas Upgrading Technologies via Enhanced Hydrogenotrophic Methanogenesis to Improve the Quality of Biogas From Anaerobic Digesters. Front. Energy Res. 8, 1–7.

Zhu, X., Chen, L., Chen, Y., Cao, Q., Liu, X., Li, D., 2019. Differences of methanogenesis between mesophilic and thermophilic *in situ* biogas-upgrading systems by hydrogen addition. J. Ind. Microbiol. Biotechnol. 46, 1569–1581.

Zotta, T., Solieri, L., Iacumin, L., Picozzi, C., Gullo, M., 2020. Valorization of cheese whey using microbial fermentations. Appl. Microbiol. Biotechnol. 104, 2749–2764.

2 Thermophilic anaerobic digestion of second cheese whey: microbial community response to H_2 addition in a partially immobilized anaerobic hybrid reactor

Abstract

In this study, we investigated thermophilic (55 °C) anaerobic digestion (AD) performance and microbial community structure, before and after hydrogen addition, in a novel hybrid gas-stirred tank reactor (GSTR) implemented with a partial immobilization of the microbial community and fed with second cheese whey (SCW). The results showed that H_2 addition led to a 25% increase in the methane production rate and to a decrease of 13% in the CH_4 concentration as compared with the control. The recovery of methane content (56%) was reached by decreasing the H_2 flow rate. The microbial community investigations were performed on effluent (EF) and on interstitial matrix (IM) inside the immobilized area. Before H_2 addition, the *Anaerobaculaceae* (42%) and *Lachnospiraceae* (27%) families dominated among bacteria in the effluent, and the *Thermodesulfobiaceae* (32%) and *Lachnospiraceae* (30%) families dominated in the interstitial matrix. After H_2 addition, microbial abundance showed an increase in the bacteria and archaea communities in the interstitial matrix. The *Thermodesulfobiaceae* family (29%) remained dominant in the interstitial matrix, suggesting its crucial role in the immobilized community and the SHA-31 family was enriched in both the effluent (36%) and the interstitial matrix (15%). The predominance of archaea *Methanothermobacter thermoautrophicus* indicated that CH_4 was produced almost exclusively by the hydrogenotrophic pathway.

2.1 Introduction

The dairy industry is one of the main sources of industrial wastewater in Europe with cheese whey (CW) and second cheese whey (SCW) making up a large part (Zotta et al., 2020). SCW is a byproduct generated from the precipitation of CW proteins by means of heat (80–90 °C) with added organic acids and salts for the production of cottage and ricotta cheeses. Similar to CW, SCW is a highly pollutant dairy waste with a significant organic load (biochemical oxygen demand (BOD) 30 g L^{-1}, chemical oxygen demand (COD) 60–80 g L^{-1}, and lactose 40–50 g L^{-1}) but with lower levels of fat (0.5–8 g L^{-1}) and protein (0.5–8 g L^{-1}) and a higher salinity (7–23 mS cm^{-1}) (Zotta et al., 2020).

The high organic loads of both SCW and CW represent severe disposal and pollution issues for the dairy industry and a huge opportunity for bioenergy and biochemicals production (Ahmad et al., 2019; Asunis et al., 2020; Carvalho et al., 2013). In particular, methane production by anaerobic digestion (AD) can be an opportunity for small-medium dairies that cannot afford the high investment costs associated with the implementation of CW valorization technology. According to Mainardis et al., biogas dairy plants that use simple low-cost digesters, can provide most of the electricity and heat necessary for the plants, improving energy balance and reducing transport and management costs (Mainardis et al., 2019). However, it is known that the high easily-fermentable organic content and low bicarbonate alkalinity of raw SCW, as well as of CW, unbalance the AD process towards an accumulation of volatile fatty acids (VFAs), and a decrease in the pH values far below the optimum value for the methanogens is often observed (Diamantis et al., 2014). Different solutions have been suggested in order to control acidification and rule out the risk of failure for the AD process such as supplementation of alkalinity during the process or into the feed, use of the codigestion with a substrate having high buffering capacity (e.g., live-stock manure or slurry and sewage sludge), and use of two-stage reactor configuration (Lembo et al., 2016; Luo e Angelidaki, 2013a; Treu et al., 2019; Vasmara e Marchetti, 2017). Although the codigestion strategy can be a sustainable option for farms, its implementation is not economically and environmentally sustainable for dairies. Furthermore, when digesting CW in combination with pathogenic waste streams, health and safety issues could discourage their use. In Europe, only 7.1% of the milk produced is processed directly on farms, while the vast majority of raw milk is delivered to dairies (Eurostat, 2019). In addition, the two-stage configuration entails a higher investment cost making it economically unattractive (Rajendran et al., 2020).

Different one-stage reactor configurations have been used for methane production from CW. Among these, the continuously stirred tank reactor (CSTR) configuration has several advantages such as reasonable control, easy operation and cleaning, minor operating cost, and high removal efficiency (Goli et al., 2019). However, a very low conversion per unit volume is obtained. Recently, Faisal et al. showed that the addition of high density polyethylene carriers to batch anaerobic digestion of agricultural wastes supported the formation of biofilm leading to significant increases in substrate utilization and biogas and biomethane production (Faisal et al., 2020). Similarly, Ramasamy et al. (2000) indicated that the incorporation of plastic media into a CSTR enhanced methane yield from dairy wastewaters more than 20% and without pH control (Ramasamy e Abbassi, 2000).

Although relevant improvements in process performances were reported, no information concerning the impact of the biomass support material on the microbial communities was reported in these studies.

Recently, Treu et al. proposed the H_2 addition in CSTR used for CH_4 production from raw CW, as a strategy to manage the low pH value of the substrate (Treu et al., 2019). The *in situ* H_2 addition, namely biogas *in situ* upgrading, is a technology generally used to increase the methane content in biogas, i.e., the introduced H_2 is combined with carbon dioxide (CO_2) produced in the process, to generate further CH_4 by the hydrogenotrophic methanogens. The diffusion of H_2 in the liquid phase is the limiting factor of this technology, i.e., due to the low H_2 solubility it is not bioavailable for microorganisms (Alfaro et al., 2019; Bassani et al., 2016; Díaz et al., 2015; Martin et al., 2013).

Moreover, the addition of H_2 could unbalance the equilibrium of the system. The additional H_2 represents an extra substrate for the microbial communities of bacteria and archaea involved in the AD process. It could cause a modification of the microbial com- munity populating the biogas reactor. In particular, if hydrogenotrophic methanogens are unable to quickly remove the H_2, its partial pressure will increase leading to disruption of the syntrophic interaction occurring between H_2 producing microorganisms (heterotrophic community as acidogenic and acetogenic bacteria) and H_2 consuming microorganisms (autothrophic bacteria as homoacetogenic bacteria and archaea as hydrogenotrophic methanogens). This condition can lead to the accumulation of propionic and acetic acids and, consequently, a decrease of pH outside the optimal range for the methanogenic archaea produces adverse effects on the whole process. In contrast, due to the removal of CO_2, inhibition of methanogenesis could be generated by an increase in pH to values above 8.5 (Angelidaki et al., 2018).

Little is known about how the H_2 addition could affect the microbial community responsible for the AD process, especially in concomitance with other operating parameters and reactors configurations.

The current study aimed to characterize a thermophilic (55°C) anaerobic digestion (AD) performance and the microbial community structure, before and after *in situ* H_2 addition, in a novel hybrid gas-stirred reactor, namely a gas-stirred tank reactor (GSTR), fed with undiluted SCW. The GSTR hybrid reactor is the result of the CSTR modification, in which about one third of the reactor's working volume was filled with polymeric supports (high-density polyethylene, HDPE) and a gas recirculation was installed for homogenization of nutrients and as a strategy to improve

H_2 solubility. The thermophilic condition was selected to exploit the acclimatization of the mixed microbial community used as inoculum and collected from a thermophilic (55°C) anaerobic digestion plant. The microbial community of the effluent and the interstitial matrix inside the immobilized area were characterized by fluorescence *in situ* hybridization (FISH) and Illumina next-generation sequences (NGS) techniques. The impacts of hydrogen addition on the performance of methane production and on the modification of the microbial community structure were investigated.

2.2 Materials and Methods

2.2.1 Substrate Characteristics

In Italy, 4.9 Mt of cheese whey are produced annually and about 1 Mt is used to produce ricotta, a typical soft cheese of the Mediterranean region, generating a second cheese whey (SCW) called "scotta". Moreover, the manufacturing of ricotta cheese is also widespread in Latin America and in USA, where it is referred to as requeson and ricottone, respectively.

SCW is periodically collected at Formaggi Boccea, a small dairy factory located in Rome, Italy, providing a thermophilic (55°C) biogas plant fed with SCW. The wastewater was stored at 20°C and thawed before use. Undiluted SCW was fed into the GSTR reactor. Due to the fluctuations of the residual organic load derived from the production process, slight variations in its composition were observed. The main physical and chemical characteristics of the SCW are presented in Table 1 and figure 2 represent an example of ricotta cheese and SCW used in this study

Table 1. Main characteristics of second cheese whey (SCW) used in this study.

Parameters	Range
pH	5.90-6.20
Lactose (gL^{-1})	40-60
Total Solid (TS) (gL^{-1})	47-64
Volatile Solid (VS) (gL^{-1})	40-54
COD (gL^{-1})	45-70
Proteins (gL^{-1})	0.45-0.90
$NH4^{+}$ (gL^{-1})	0.10-0.12
Total Volatile Fatty Acids (TVFAs) (gL^{-1})	1.5-2.5

Figure 1. Example of ricotta cheese and second cheese whey (SCW).

2.2.2 Reactors Setup and Operation

The experiment was carried out in a novel hybrid gas-stirred tank reactor (GSTR) with a working volume of 49 L, at thermophilic temperature (55± 1°C) and atmospheric pressure. Figures 2 and 3 shows the scheme and a picture of the reactor and the overall equipment design. Reactor mixing and homogenization of nutrients was ensured by continuous gas recirculation. A vacuum pump took the biogas from the reactor headspace and injected it into the bottom of the GSTR. Biofilm media carriers were used as packing material (Scubla MBBR 800, HDPE) with a specific surface area of 800 m^2/m^3. The immobilized area was completely submerged in the middle part of the reactor and occupied a volume of 15 L. Media carriers were enclosed in a mesh bag to prevent washing out in the outlet stream. Biogas coming from both gas recirculation and gas outlet lines were dehumidified by a chiller and the condensed water was returned into the digester. The GSTR was inoculated with thermophilic (55°C) sludge obtained from the Formaggi Boccea biogas plant. Undiluted second cheese way (SCW) was fed in a continuously way from the bottom using a peristaltic pump. The hydraulic retention time (HRT) was 15 days, and the organic loading rate (OLR) was on average within a range of 2.18 ± 0.14 $g_{COD}\ L^{-1}\ d^{-1}$ and 2.4 ± 0.12 $g_{COD}\ L^{-1}\ d^{-1}$.

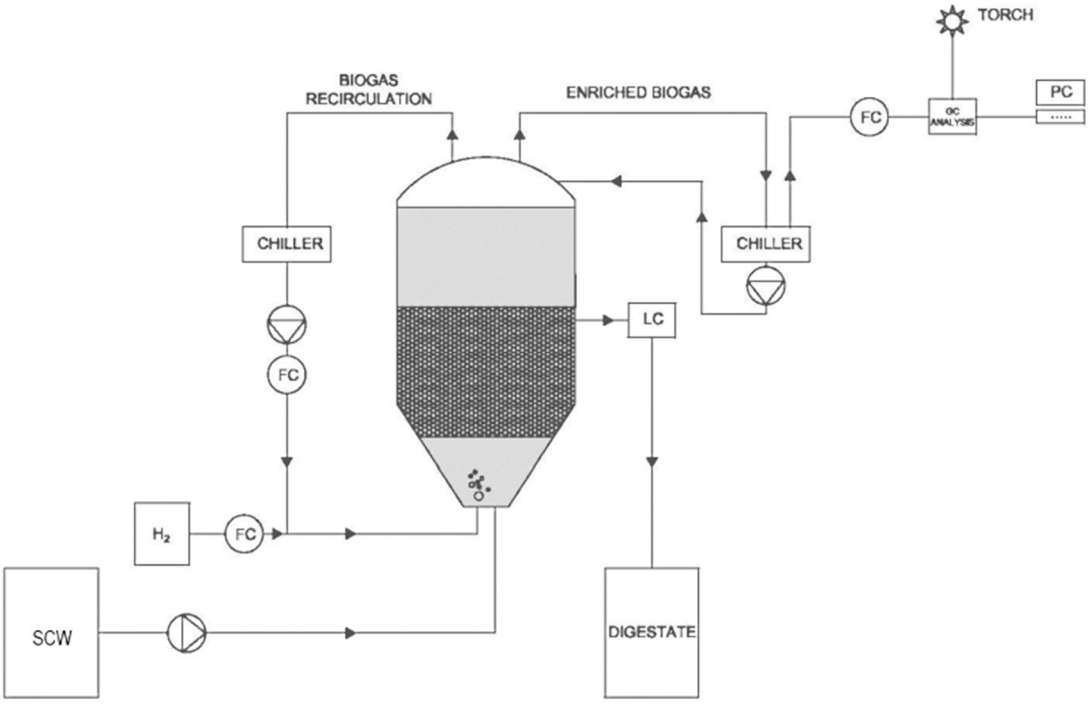


Figure 2. Schematic representation of the hybrid gas-stirred tank reactor (GSTR) and the experimental equipment. LC, liquid control; FC, flow control; SCW, second cheese whey.

Figure 3. Hybrid gas-stirred tank reactor (GSTR) employed in the experiment.

Initially, a biogas recirculation flow rate of 59 L L_r^{-1} d^{-1} was applied. When a stable AD

process was achieved, pure H_2 (99%) obtained from an electrolyzer (DBS, model PG-H_2 100), was continuously injected into the reactor from the bottom by the biogas recirculation line. In order to avoid an increase in hydrogen partial pressure, the stoichiometric ratio H_2/CO_2 used in this experimental phase was lower (2.7:1 UP1 and 2:1 for UP2, UP for upgrading phase) than needed for hydrogenotrophic methanogenes (4:1). The starting H_2 flow rate was 1.8 L_{H_2} L_r^{-1} d^{-1} and gas recirculation flow rate was maintained at 59 L L_r^{-1} d^{-1}.This experimental phase is denoted as Upgrading 1 (UP1). Thereafter, the H_2 flow rate was decreased to 1.32 L_{H_2} L_r^{-1} d^{-1} and gas recirculation flow was increased to 118 L L_r^{-1} d^{-1}. This experimental phase is indicated as UP2. Both UP1 and UP2 lasted for 30 days.

2.2.3 Analytical Methods

H_2, CH_4, CO_2, N_2, and O_2 percentages in biogas were analyzed by online Micro Gas Chromatograph Varian (GC4900). Two columns of 10 m were used. The first, MS 5A, had a stationary phase with molecular sieves capable of separating the permanent gases of low molecular weight, H_2, CH_4, N_2, and O_2. The second column was a Poraplot U, used as a stationary phase divinyl benzene able to separate CO_2. Argon was used as the carrier gas. The biogas and H_2 flow rates were monitored by digital online flow meters (EL-Flow select series, Bronkhorst High-Tech B.V, Ruurlo Netherland).The flows of the different gases were calculated on the basis of the percentage compositions of the individual gas present in the biogas. Volatile fatty acids (VFAs), lactic acid, alcohols, and sugars were analyzed by a HPLC Thermo Spectrasystem, equipped with a UV detector (λ = 210 nm)and a refraction index detector, using the isocratic method of analysis at 75°C with Column Rezex ROA-Organic Acid H^+ (8%), size 300 × 7.8 mm Phenomenex, USA.

2.2.4 Calculation

Performance and efficiency of the methanation process were expressed as the methane evolution rate (MER), i.e., the rate of CH_4 production from the injected H_2, and H_2 conversion efficiency (η_{H_2}). The MER (Equation (1) expresses the increase in the specific CH_4 production rate (L_{CH_4} L_r^{-1} d^{-1}) caused by H_2 injection as compared with the CH_4 production rate in the control condition. It is calculated as follows (Equation (1)):

$$MER = CH_{4UPs} - CH_{4AD} \quad (1)$$

where UPs are the upgrading periods (with H_2 addition) and AD is the control period (with- out H_2) The H_2 gas-liquid mass transfer rate, namely r_t (L_{H_2} L_r^{-1} d^{-1}), and the efficiency of H_2 utilization, namely η_{H_2} (%), were calculated according to Equations (2) and (3), respectively:

$$R_t = H_2 \text{ in flow rate} - H_2 \text{ in output gas} \quad (2)$$

$$\eta H_2 = \frac{H2in\ Flow\ rate - H2in\ uotput\ gas}{H2\ in\ Flow\ rate} * 100 \quad (3)$$

Where H_2 in flow rate and H_2 in output gas are the volumetric H_2 flows entering and leaving the reactor. It was assumed that all the H_2 transferred to the liquid phase was ultimately converted to CH_4 or used for other microbial metabolic pathways or employed for microbial growth (Alfaro et al., 2019; Díaz et al., 2015).

The H_2 rate converted to biomethane (L L_r^{-1} d^{-1}) was calculated according to Equation (4):

$$\text{Rate to biomethane} = 4 \times (CH_4 \text{ in UP output gas} - CH_4 \text{ in AD output gas}) \quad (4)$$

Where 4 is the stoichiometric coefficient according to Equation (4) $H_2 + CO_2 = CH_4$. CH_4 in UP output gas (L_{CH4} Lr^{-1} d^{-1}) is the rate of CH_4 produced in UP experimental phases, and CH_4 in AD output gas (L_{CH4} Lr^{-1} d^{-1}) is the rate of CH_4 produced in AD phase.

2.2.5 Samples collection for microbial community analysis

Samples for microbial community analysis were collected at the end of AD and UP phases from both the liquid medium and the interstitial matrix between the HDPE supports. After the AD phase, the GSTR was opened to collect the interstitial matrix; its dark-grey color and the presence of flocs indicated the formation of a biofilm. Before the UP phases, the GSTR was flushed with nitrogen to restore the anaerobic condition and was operated under the same previous operating conditions until N_2 was no longer detected. Samples were collected in duplicate, aliquoted (10 mL) and stored at 20°C for different uses (i.e., DNA extraction and fluorescence *in situ* hybridization (FISH) analysis). They are recognized as EF-AD or EF-UP2 (AD, anaerobic digestion phase; UP2, H_2 upgrading at the end of UP2 phase; EF, effluent,) and IM-AD or IM-UP2, (IM, interstitial matrix).

2.2.6 Illumina Sequencing

Genomic DNA was extracted using a Gene MATRIX Bacterial and Yeast Genomic DNA Purification Kit (EURxLtd. Gdansk Poland), in accordance with the manufacturer's recommendations. The quantity and quality assessment of the extracted DNA were performed using spectrophotometry (Eppendorf BioPhotometer plus). All DNA samples were stored at 20°C until use.

Library preparation was performed utilizing a 460bp amplicon corresponding to the hypervariable V3-V4 region of 16S rRNA using universal primers (S-D-Bact-0341F and S-D-Bact-0785R for bacteria and archaea domains). Sequencing was performed at the Department of Agricultural Sciences, University of Naples Federico II (Portici, Italy) using the Illumina MiSeq platform. From the generated reads, demultiplexing, quality filtering, trimming, merging, and operative taxonomical units (OTUs) picking were performed using QIIME pipeline (Caporaso et al., 2010).

The taxonomical assignment was performed with the Greengenes database at a 97% similarity. The bioinformatics analysis of all sequences generated more than 220,000 reads, with an average of 26,800 counts per sample. The results reported are focused on the most abundant members of the microbial community having a relative abundance $\geq$ 0.5%. Raw sequences data were deposited at the Sequence Read Archive (SRA) under the BioProject PRJNA681387 with the accession numbers SAMN1695146 and SAMN16951461. Shannon–Weaver (H) and Pielou's evenness (E) indices were calculated using the relative abundance of sequences obtained from NGS sequencing at the family level.

2.2.7 Total Microbial Abundance and Bacteria and Archaea Detection

DAPI fluorescent staining and fluorescence *in situ* hybridization (FISH) were used to evaluate the microbial abundance and bacteria and archaea relative abundance, respectively. The collected samples were fixed (4% w/v paraformaldehyde fixative solution for 6 h, at 4°C), as described in Amann, 1990, and stored (20°C) (R. I. Amann et al., 1990) until use. In order to detach microbial cells from inorganic particles, a cleaning sample procedure was performed immediately after thawing and before analysis, using the density gradient medium OptiPrep™ (Axis-Shield PoCAs, Oslo Norway) (Barra Caracciolo et al., 2005). Total microbial abundance (cells mL^{-1}) was determined in the effluent and interstitial matrix by direct cell counting after DAPI staining (4′,6-diamidino-2-phenylindole) (1 µg mL^{-1}) (Barra Caracciolo

et al., 2005) using the epifluorescence microscopy AXIOSKOP 40 (Carl Zeiss, Germany) equipped with a ZEISS HXP 120v Light Source and 1000 magnification.

The FISH analysis (in triplicate) was carry out using samples collected from effluent and interstitial matrix focusing on the relative abundance (%) of archaea and bacteria. Analyses were performed as described in Amann et al., 1995 (R. Amann et al., 1990) and oligonucleotide probes for the Bacteria (EUB338 II,III) and Archaea (ARC915) domains were used (R.I. Amann, K.H. Ludwig, 1995).

The oligonucleotide probes (50 ng μL^{-1}), labeled with carboxyfluoresce in (FAM) or indocarbocianine (Cy3) dyed at the 5′-end, were purchased from MWG AG Biotech,

Germany. All the hybridizations were carried out in combination with DAPI staining to estimate the proportion of cells targeted by the specific probes out of the total cells. Slides were mounted with a few drops of Vecta Shield (Vector Laboratories, USA) and seen using Zeiss epifluorescence microscope. The average of cells for each target probe (15 fields for each slide) was used to evaluate the percentage of the positive signals versus DAPI-positive cells. Microbial abundance of bacteria and archaea in the samples (cells mL^{-1}) was calculated using the relative abundances obtained by FISH technique and the microbial abundance detected after DAPI staining.

2.3 Results

2.3.1 Reactor Performance

The daily course profiles of process performances of all experimental phases are shown in Figure 4 and the steady operation condition process performance data are summarized in Table 2.

During the AD period, starting from the 10th day to the end of this phase, a stable methane production rate of 0.79±0.04 $L_{CH4}\ L_r^{-1}\ d^{-1}$ was reached. The pH of the reactor was around 7, indicating a strong buffer capacity of the system, although no pH control was applied. The average CH_4 content in the biogas was 55.5 ± 0.8% and the average specific methane yield (SMY) was 0.329 ± 0.02 $L_{CH4}\ g_{COD}^{-1}$, which corresponded to 94% of the maximum theoretical value obtainable by the conversion of organic matter into methane (i.e., 0.350 $L_{CH4}\ g\ _{COD}^{-1}$) (Angelidaki et al., 2018; Treu et al., 2019). The substrate was almost completely consumed, as confirmed by the low average value of lactose (270 ± 28 mg L^{-1}) detected in the reactor effluent, indicating an efficient performance of the AD process. The acetic acid was the unique VFA found during the

AD process, moving from an average value of 210 ± 5.6 mg L^{-1} during the first 10 days to that of 37 ±2.4 mg L^{-1} at the end of AD. Moreover, in order to verify the effect of biogas recirculation on the soluble e metabolite distribution along the GSTR, an HPLC analysis was also performed on the lower and upper liquid fractions. The average lactose and acetic acid concentrations of 270 ± 28 mg L^{-1} and 45 ± 10 mg L^{-1}, respectively, were measured indicating a good mixing of the reactants.

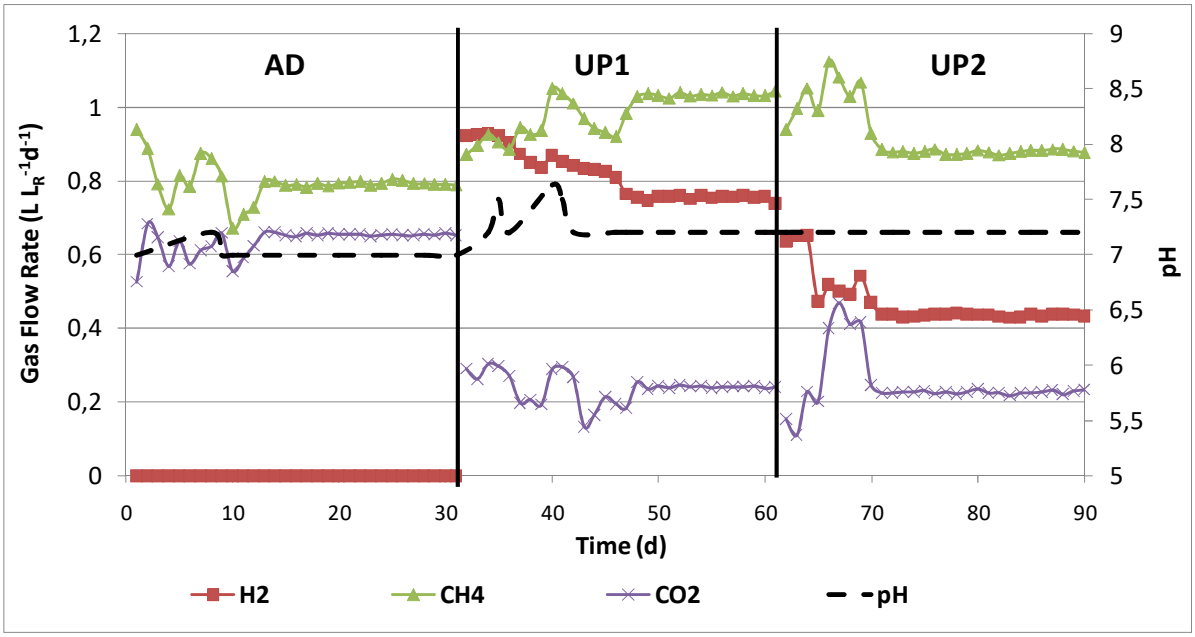


Figure 4. Daily course profiles of process performances during the three experimental phases.

Table 2. Process performances at the end of the three experimental phases.

Parameters	AD	UP1	UP2
OLR (g COD $L_r^{-1}d^{-1}$)	2.4 ± 0.12	2.18 ± 0.14	2.28 ± 0.25
H_{2in} flow rate (L L_r^{-1} d^{-1})	-	1.76 ± 0.01	1.32 ± 0.01
Biogas recirculation rate (L L_r^{-1} d^{-1})	59 ± 5	59 ± 5	118 ± 5
Biogas production rate (L L_r^{-1} d^{-1})	1.42 ± 0.06	2.05 ± 0.07	1.65 ± 0.18
H_2%	-	39.7 ± 3.0	28.4 ± 2.7
CH_4%	55.5 ± 0.8	48.2 ± 3	56.0 ±1.9
CO_2%	45.1 ± 1.65	11.6 ± 1.62	14.8 ± 3.07
H_2 Flow rate (L L_r^{-1} d^{-1})	-	0.81 ± 0.06	0.47 ± 0.07
CH_4 Flow rate (L L_r^{-1} d^{-1})	0.79 ± 0.04	0.99 ± 0.06	0.92 ± 0.08
r_t (L L_r^{-1} d^{-1})	-	0.95	0.85
η_{H2} (%)	-	54	65
Specific methane yield (SMY) (L_{CH4} g COD^{-1})	0.329 ± 0.02	-	-
MER (L_{CH4}*$L_r^{-1}d^{-1}$)	ND	0.20	0.13
Lactose (mg L^{-1})	270 ± 28	225 ± 72	209 ± 10
Acetic acid (mg L^{-1})	45 ± 10	53 ± 19	38 ± 5.2

During the AD period, starting from the 10th day to the end of this phase, a stable methane production rate of 0.79 ± 0.04 $L_{CH4}\ L_r^{-1}\ d^{-1}$was reached. The pH of the reactor was around 7, indicating a strong buffer capacity of the system, although no pH control was applied. The average CH_4 content in the biogas was 55.5 ± 0.8% and the average specific methane yield (SMY) was 0.329 ± 0.02 $L_{CH4}\ gCOD^{-1}$, which corresponded to 94% of the maximum theoretical value obtainable by the conversion of organic matter into methane (i.e., 0.350 $L_{CH4}\ g\ COD^{-1}$) (Angelidaki et al., 2018; Treu et al., 2019). The substrate was almost completely consumed, as confirmed by the low average value of lactose (270 ± 28 mg L^{-1}) detected in the reactor effluent, indicating an efficient performance of the AD process. The acetic acid was the unique VFA found during the AD process, moving from an average value of 210 ± 5.6 mg L^{-1} during the first 10 days to that of 37 ± 2.4 mg L^{-1}at the end of AD. Moreover, in order to verify the effect of biogas recirculation on the soluble metabolite distribution along the GSTR, an HPLC analysis was also performed on the lower and upper liquid fractions. The average lactose and acetic acid concentrations of 270 ± 28 mg L^{-1} and 45 ± 10 mg L^{-1}, respectively, were measured indicating a good mixing of the reactants.

This result is among the highest SMY obtained from AD of synthetic or raw dairy wastewaters in hybrid anaerobic biofilm reactors (0.03–0.359 $L_{CH4}\ gCOD^{-1}$) (Goli et al., 2019; Karadag et al., 2015). However, these studies were conducted at mesophilic temperature. So far, there have been few studies on single-stage thermophilic anaerobic digestion using only dairy wastewaters (Asunis et al., 2020). Yang et al. achieved a maximum yield of 0.360 $L_{CH4}\ g\ _{COD}^{-1}$using a 5L CSTR reactor under an HRT value of 7.5 days and using diluted CW (10g $_{CO}$D L^{-1}) (Yang et al., 2003). Other authors have recorded an unstable AD process with poor thermophilic (55 °C) CH_4 production yield (0.100 $L_{CH4}\ g\ _{COD}^{-1}$ ± 0.21) and a remarkable accumulation of acetic acid 10 ± 1 g L^{-1} using a 3L CSTR configuration with an OLR of 2.4 g $_{COD}L^{-1}\ d^{-1}$ and an HRT of 15 days using cheese whey permeate and cheese waste powered as substrates (Fontana et al., 2018b). Fernandez et al., obtained a SMY of 0.315 ± 6.6 $L_{CH4}\ g\ _{COD}^{-1}$ during a thermophilic AD of deproteinized CW in a 25 L anaerobic sequencing batch reactor (ASBR) at an HRT of 8.3 days and an OLR of 4.6 ± 0.3 $g_{COD}\ L^{-1}\ d^{-1}$ (Fernández et al., 2015). Although different HRT and OLR values were used, the yields obtained were comparable to ours, suggesting that the GSTR, used in this study, maintained the characteristics of a CSTR for the homogenization of nutrients in the liquid phase. However, the implementation of supports for the immobilization of the biomass makes it comparable to an ASBR whose peculiar feature is to uncouple the SRT (solid retention time) from the HRT.

The UP1 started at day 30 and, after 12 days, a stable methane production process was reached and lasted until the end of the experimental phase (60 days). During the steady state of UP1, the CH_4

production rate was 0.99 ± 0.06 $L_{CH4}\,L_r^{-1}\,d^{-1}$, which represented an increase of 25% as compared with the AD phase. The calculation of the efficiency of H_2 utilization (ηH_2), suggests that 54% of H_2 added is utilized by the microbial community.

However, the incomplete H_2 consumption led to a dilution of CH_4 concentration as compared with that obtained in AD, indeed the composition of the outflow biogas in the UP1 phase was 48.2% CH_4, 39.8 H_2, and 11.6% CO_2. Considering the high unconverted percentage of H_2 observed in UP1, it was assumed that the H_2 injection rate of 1.8 $L_{H2}\,L_r^{-1}\,d^{-1}$ used in UP1 was too high. Therefore, the H_2 inlet flow was decreased to 1.32 $L_{H2}\,L_r^{-1}d^{-1}$ in the UP2 phase. Moreover, in order to increase the H_2 dissolution in the liquid phase, the gas recirculation rate was increased from 59 to 118 $L_{H2}\,L_r^{-1}d^{-1}$.

During the steady state of UP2 (reached after day 10) the CH_4 flow rate was equal to 0.92 ± 0.08 $L_{CH4}\,L_r^{-1}\,d^{-1}$ and the percentage of CH_4 in the biogas went up to 55%. The pH increased, reaching a value of 7.3–7.4. The CH_4 production rate was 17% higher as compared with AD and no accumulation of acetic acid (38 ± 5 mg L^{-1}) or lactose (208 ± 10.5 mg L^{-1}) were observed. The H_2 efficiency value increased to 65%, but the MER value (0.13 $L_{CH4}\,L_r^{-1}\,d^{-1}$) was lower than the one calculated in UP2 (0.20 $L_{CH4}\,L_r^{-1}\,d^{-1}$). According to the stoichiometric reaction $4H_2 + CO_2 = CH_4+2H_2O$, MER values of 0.21 and 0.24 $L_{CH4}\,L_r^{-1}\,d^{-1}$were expected, respectively.

These differences could be partly explained by the use, in UP1 and UP2, of OLRs slightly lower than the one used in AD, involving in an overestimation of the CH_4 formation rate from SCW. In addition, taking into account that the amount of acetate as H_2 equivalent can be neglected, two further metabolic pathways of the H_2 added other than the production of CH_4 took place inside the GSTR reactor. For example, during the methanation process, a distinct part of both H_2 and CO_2 are metabolized to produce biomass by the archaea community (Lecker et al., 2017). The results of the FISH analysis seem to confirm this hypothesis; the archaea abundance was more than doubled after the H_2 dispersion phase in EF-UP2 and it increased in IM-UP2. However, sulphate-reducing bacteria also utilize H_2 as a substrate to reduce sulphate to hydrogen sulphide. In this study, the hydrogen sulphide content of biogas was not monitored.

Briefly, although the purpose of this study was not to improve the efficiency of the *in situ* biomethanation process and, although higher CH_4 concentration values were achieved in a published paper, the above results demonstrated that it was possible to convert H_2 to CH_4 in this novel GSTR with partially immobilized biomass by using HDPE supports.

2.3.2 Total Microbial Abundance and Bacteria and Archaea Detection

Total microbial abundance (DAPI staining), as well as abundance in bacteria and archaea (FISH technique associated with DAPI staining) under different experimental conditions are shown in Figure 5

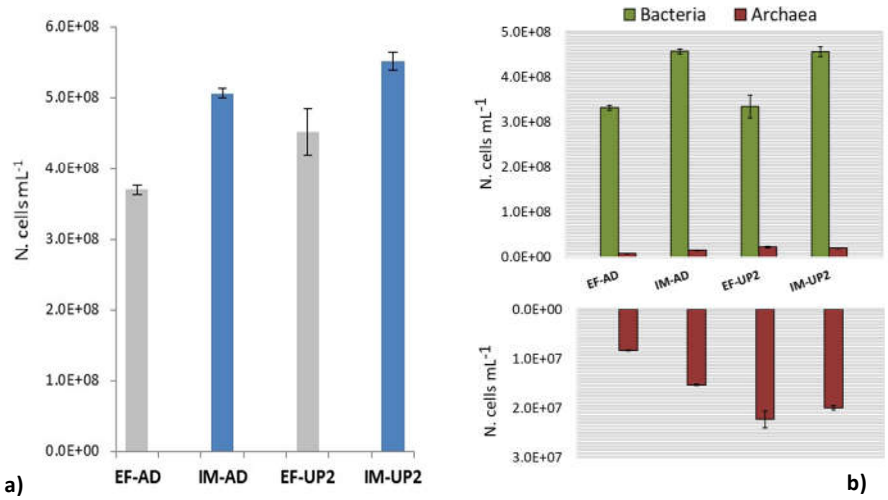


Figure 5. Microbial abundance (N. cells mL^{-1}) under different experimental conditions is reported.(a) Total microbial abundance detected in the effluent (EF, gray bars) and in the interstitial matrix (IM, blue bars), before (AD, anaerobic digestion) and after (UP2) H_2 addition, using DAPI staining; (b)Abundance of bacteria (green) and archaea (red) in the effluent (EF) and interstitial matrix (IM), before and after H_2 addition (AD and UP2, respectively) obtained by FISH technique and DAPI staining.

The highest and the lowest total microbial abundances were detected, respectively, in IM-UP2 with 5.53E+08 ± 1.25E+07 cells mL^{-1} and in EF-AD with 3.70E+08 ± 6.24E+06cells mL^{-1} (Figure 5a). In both effluent (EF) and interstitial matrix (IM), the total microbial abundance increased after H_2 addition by 22% and 9%, respectively. Both IM-AD and IM-UP2 had a total microbial abundance higher than the corresponding effluent phase (EF-AD and EF-UP2) by 37% and 22%, respectively, highlighting the positive effect that the presence of immobilizing supports exerted on the microbial community. The FISH investigations showed a strong predominance of bacteria in the microbial communities of all experimental conditions (Figure. 5b), while archaea contribution to the microbial communities, ranged between 2.2% and 5.2%, in line with the concentration usually detected in microbial communities during an AD process (Pervin et al., 2013). More specifically, before H_2 addition the bacterial cells detected were 89% and 91% in EF-AD and IM-AD, respectively, while at the end of UP2 phase their concentration decreased to 80% and 84%, respectively. The H_2

addition positively affected archaea populations, especially in EF-UP2 effluent samples, for which a concentration of 5.2% corresponding to 2.23E+07 cells mL^{-1} of archaea was detected, with an increase of 134% as compared with EF-AD which showed a concentration of 0.83+07 cells mL^{-1} (Figure 5b). The percentage of archaea in the interstitial matrix, before and after H_2 addition, was 3.1% and 3.7%, respectively, corresponding to a 43% increase in the interstitial matrix after the H_2 addition. Moreover, the presence of HDPE supports generated a favorable habitat for the microbial community, as evidenced both by the total microbial (Figure 5a) and bacteria abundances (Figure 5b) in both experimental phases. After the H_2 addition, an increase in total microbial (Figure 5a) and archaea abundances (Figure 5b) in the interstitial matrix was observed. It can be hypothesized that these supports, inserted in the GSTR to slow down the flow of H_2, promote its contact with microbial cells and also provide a habitat refuge in which the disturbance of the spatial disposition of AD populations with different cell leaching have been limited. Other authors (Ferraro et al., 2020) observed an increase in the archaea component when a porous support was offered to a mesophilic anaerobic microbial community as a refuge habitat, although the present study is among the first to refer to a thermophilic condition.

2.3.3 Illumina Sequencing

The microbial communities were analyzed by Illumina-based 16S sequencing. The composition of microbial community at the different taxonomic levels tended to decrease from 96% of phyla toward 25% of species level, showing values of 85% and 50% at families and genera level, respectively. The low assignment at genera and species levels suggested the presence, in the microbiome, of numerous unexplored or undescribed taxa.

2.3.4 Bacteria Communities

The relative abundance of *Bacteria* at the phylum level is shown in Figure 6.

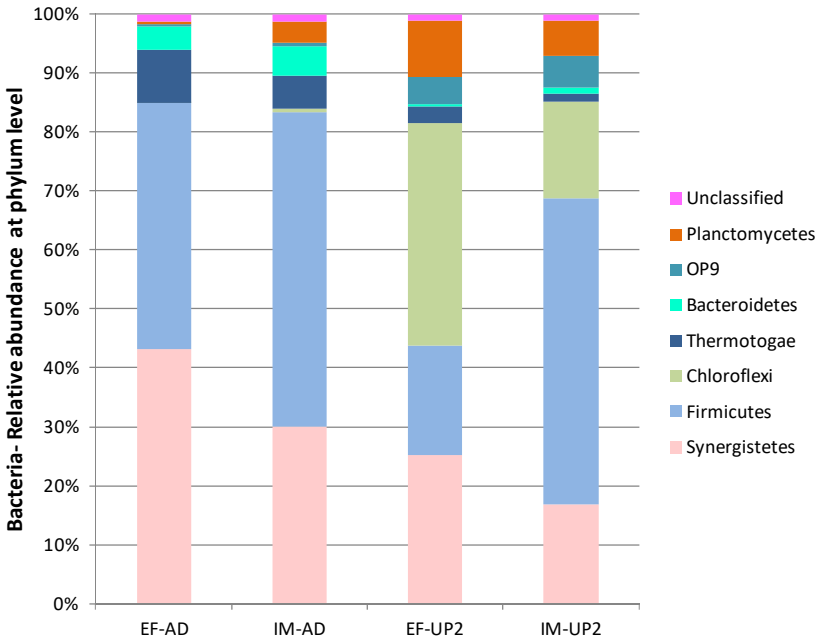
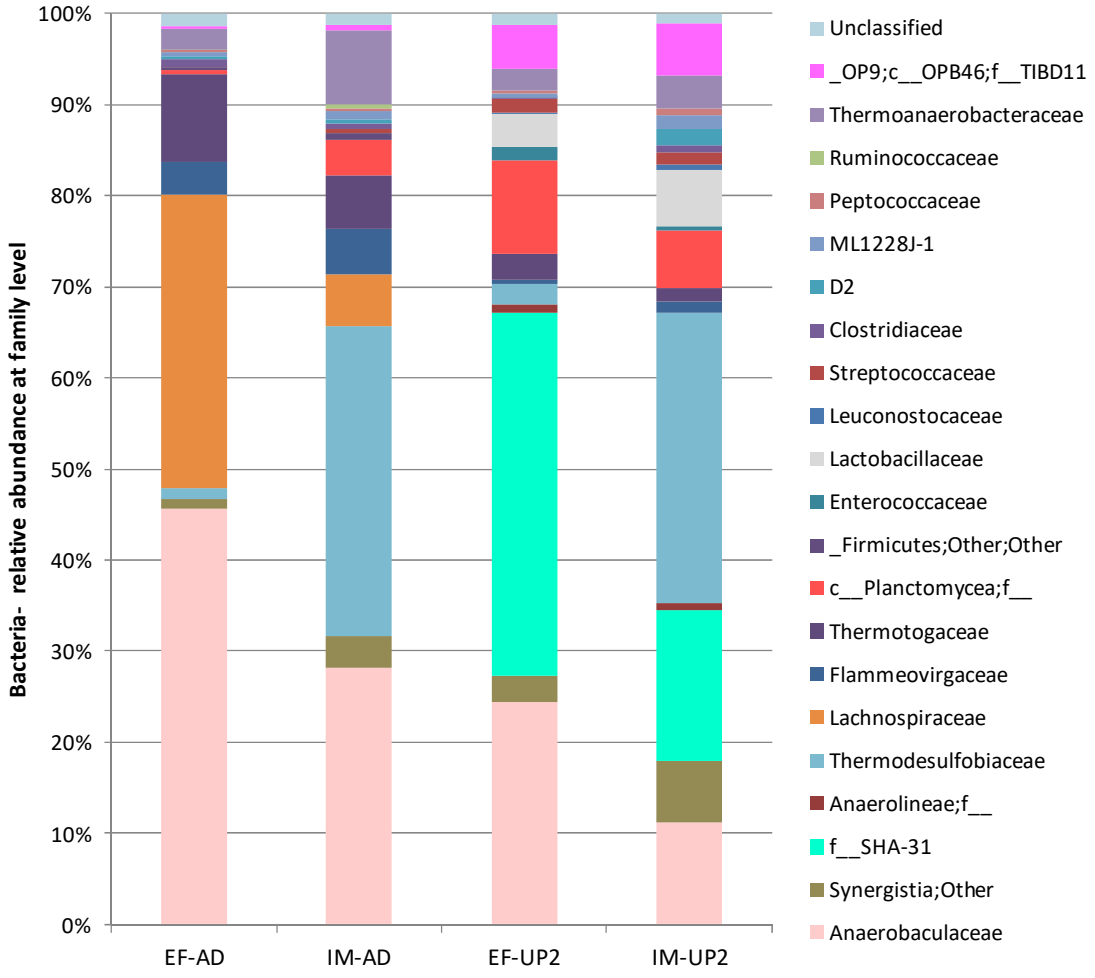


Figure 6. Relative abundances of the bacteria community at the phylum level at the end of AD (anaerobic digestion) and UP2 (H_2 injection) phases. Communities with a relative abundance ≥ 0.5% (in at least one sample) are reported. EF, effluent and IM, interstitial matrix.

The microbial communities during the AD phase were dominated by *Synergistetes* (43% and 30% in EF and IM, respectively) and *Firmicutes* (42% and 53% in EF and IM, respectively). Representatives of *Thermotogae* and *Bacteriodetes* phyla were also abundant in microbial communities of the AD phase, representing 9% and 4% in the EF sample and 6% and 5% in the IM sample, respectively. Minor representative phyla in the AD phase were identified only in the IM sample, i.e., *Planctomycetes* (4%), *Chloroflexi* (1%) and OP9 (1%). After H_2 injection, relevant changes in the microbial community structures were observed both for effluent and interstitial matrix samples. The *Synergistetes* and *Firmicutes* phyla still represented a considerable fraction of UP2 communities, although their relative abundance decreased considerably as compared with the AD samples. In particular, in the EF-UP2 samples, *Synergistetes* represented 25% of the bacterial community, while *Firmicutes* represented 19%. A different trend was observed in the IM-UP2 sample, in which the abundance of *Synergistetes* further decreased to 17%, while the *Firmicutes* accounted for 51% of the whole community. The high increase in the relative abundance of *Chloroflexi* phylum was the most relevant observed change affecting the microbial community

structure during the UP2 phase. Representatives of this phylum accounted for 37% and 16% of the bacterial community in EF-UP2 and IM-UP2 samples, respectively. Moreover, an increase in the relative abundances of *Planctomycetes* (9% and 6% in EF and IM, respectively) and *OP9* (5% and 5% in EF and IM, respectively) was also observed. In contrast, the relative abundances of *Thermotogae* and *Bacteroidetes* decreased, representing 3% and 1.5% in EF-UP2 and 0.5% and 1% in IM-UP2 samples, respectively.

The detailed results of the bacterial communities at the family level are shown in Figure 7.



Index	EF-AD	IM-AD	EF-UP2	IM-UP2
H'	1,62	2,01	2,06	2,38
E	0,55	0,68	0,65	0,75

Figure 7. Relative abundances of the bacteria community at the family level at the end of AD (anaerobic digestion) and UP2 (H_2 injection) phases. Communities with a relative abundance ≥0.5% (in at least one sample) are reported. EF, effluent and IM, interstitial matrix. Table shows Shannon–Weaver (H') and Pielou's evenness (E) diversity indices.

Anaerobaculaceae was the unique family within *Synergistetes* detected in all samples, regardless of the experimental phase or the sampling point, thus, suggesting its functional role in the anaerobic digestion process of SCW. This family was characterized at the species level as *A.hydrogeniformans*, a moderately thermophilic NaCl requiring fermentative bacterium, attesting that it was suitable for the saline and the milk derived substrates. The same microorganism was identified by Treu et al. (2019) during thermophilic anaerobic digestion of cheese whey. Among the *Firmicutes* phylum, the highest number of families was identified. In particular, members of the *Lachnospiraceae* family were dominant in the EF-AD sample (30%) and had a lower relative abundance in the IM-AD (5%) sample. This family was not detected in the UP2 samples. Moreover, members of the *Thermoanaerobacteraceae* family were detected with a higher relative abundance in the IM-AD sample (8%) than in the EF-AD (2%) sample. These microorganisms were stable in EF-UP2 sample (2%) but showed a relevant decrease in the IM-UP2 sample (3%) as compared with the IM-AD sample. The genus *Thermacetogenium* was identified as a unique genus detected in all samples; it is characterized as an acetate-oxidizing syntrophic microorganism (Hattori, 2008). Members of the *Thermodesulfobiaceae* family were dominant in the interstitial matrix of both the AD and UP2 phases, accounting for 32% and 29% of the microbial community, respectively. Previous studies have highlighted how these families carried out an acidogenic hydrolytic activity (Bassani et al., 2015; De Francisci et al., 2015). *Coprothermobacter* was identified among the *Thermodesulfobiaceae* family as the dominant genus with a relative abundance of 32% and 29% in samples IM-AD and IM-UP2, respectively. It is worth noting that its presence in anaerobic digesters is often related to configurations using biofilm supports (Tandishabo et al., 2012). *Coprothermobacter* is a bacterial genus that includes anaerobic thermophilic members that are proteolytic and produce acetate, H_2, and CO_2. Moreover, its involvement in the syntrophic acetate oxidization (SAO) pathway during thermophilic AD processes has been previously suggested (Gagliano et al., 2015; Ho et al., 2014). A new phylogenetic affiliation has been proposed for this taxa according to its overall phenotypic properties as well as to a phylogenetic analysis supporting its placement in a distinct deeply rooted novel phylum (Pavan et al., 2018). The SHA-31 family of the class of *Anaerolineae* was revealed among the *Chloroflexi* phylum as the unique member characteristic of the UP2 experimental phase. Relative abundances corresponding to 36% and 15% of the microbial community were found in EF and IM samples of UP2 phase, respectively. Several authors (Nakasaki et al., 2020; Narihiro et al., 2012; Xia et al., 2016) considered members of the *Anaerolineae* family to be a “semi-syntrophic” microorganisms in anaerobic systems due to their involvement in heterothrophic carbohydrate degradation and in interspecies electron transfer mechanism in mutualistic cooperation with methanogens.

According to both Shannon–Weaver (H') and Pielou's evenness (E) indices, the IM-UP2 sample showed the highest diversity (H' = 2.38 and E = 0.75), suggesting that the H_2 injection had a positive impact in the presence of HDPEs supports. Moreover, a comparison of indices between EF-AD (H' = 1.62 and E = 0.55) and EF-UP2 (H' = 2.06 and E = 0.65) highlighted that microbial diversity was increased also in the effluent by the hydrogen addition. At the same time, the higher diversity in the IM-AD sample (H' = 2.01 and E = 0.68) as compared with the EF-AD sample (H' = 1.62 and E = 0.55) indicated that the immobilization strategy was also able to increase the microbial diversity.

2.3.5 Archaea Communities

Archaea microbial communities were represented by the unique phylum of *Euryarchaeota.* The overall relative abundances of all methanogens were very low during the AD phase, ranging from 0.1% in EF-AD to 0.40% in the IM-AD. Subsequently, during H_2 addition, the methanogenic population increased up to 0.64% in the effluent and up to 1.42% in the interstitial matrix. Figure 8 shows the relative abundances of *Archaea* at the family level.

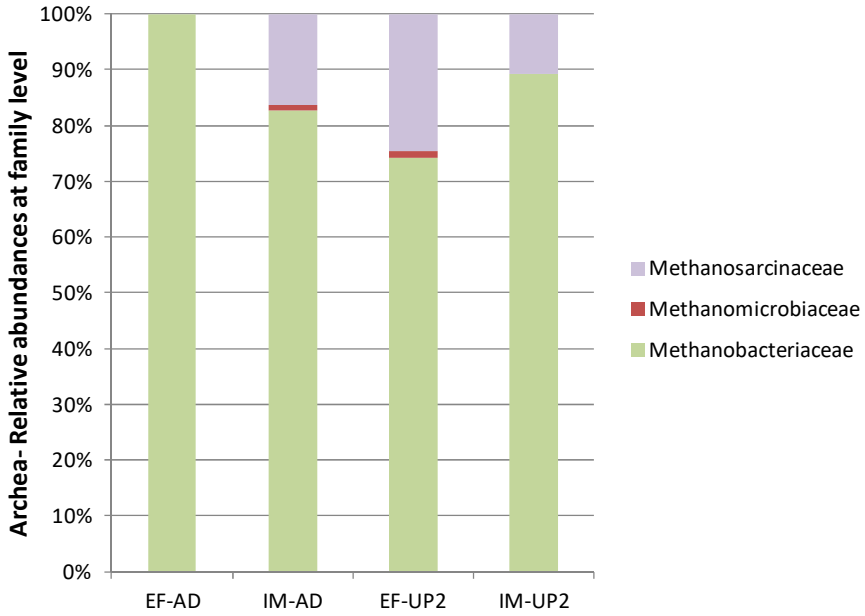


Figure 8. Relative abundances of the archaea community at the family level at the end of AD (anaerobic digestion) and UP2 (H_2 injection) phases. Communities with a relative abundance ≥0.5% (in at least one sample) are reported. EF, effluent and IM, interstitial matrix. . Table shows Shannon–Weaver (H') and Pielou's evenness (E) diversity indices

Members of *Methanobacteriaceae* family dominated in both experimental phases, as well as in effluent and interstitial matrix, showing values of relative abundances higher than 74%. Among this family, the hydrogenotrophic *Methanothermobacter thermoautrophicus* was found to be highly abundant in the AD and UP2 phases. The dominance of the hydrogenotrophic pathway during

anaerobic digestion of cheese whey was reported by Treu who suggested that this pathway was driven by the saline characteristics of a substrate (Treu et al., 2019). Moreover, members of the *Methanosarcina* family were detected in all samples except for the EF-AD sample. In particular, the relative abundance in EF-AD was 16%, increasing in EF-UP2 (25%) and lowering in IM-UP2 (11%). *Methanosarcina* are known as generalist methanogenic archaea that are able to use hydrogen/dioxide carbon, acetic acid, and methylamines (Zabranska e Pokorna, 2018) as substrates. Therefore, addition of an extra substrate, namely hydrogen, caused the enrichment of the *Methanosarcina* community.

2.4 Conclusions

The partially immobilized gas-stirred tank reactor (GSTR) used in this study was suitable for thermophilic anaerobic digestion (AD) of undiluted second cheese whey (SCW). A methane yield of 0.329 ± 0.17 L_{CH4} $gCOD^{-1}$, corresponding to 94% of the maximum theoretical value, was obtained without pH control. Moreover, H_2 injection (UP1 phase) increased the CH_4 production rate by 25%as compared with that obtained during conventional AD (AD phase) without compromising the efficiency of organic matter removal.

Data on microbial abundance showed an increase in bacteria communities at the end of both the AD and UP2 experimental phases in the interstitial matrix. Moreover, the H_2 addition (UP2) increased the archaea communities in both effluent and interstitial matrix as compared with the AD phase. From a functional point of view, we identified a common microbial ”core” in all samples represented by members of the *Anaerobaculaceae* family, strictly correlated to the SCW metabolism. In addition, in samples collected from the interstitial matrix at the end of both experimental phases, a new microbial core was identified in members of the *Thermodesulfobiaceae* family. Finally, H_2 addition caused the enrichment of a peculiar microbial core represented by members of the SHA-31 family. A common core of the methanogenic microbial community was identified in the hydrogenotrophic members of the *Methanobacteriaceae* family in AD and UP experimental phases. Moreover, the versatile members of *Methanosarcinaceae* were enriched by the H_2 addition. All these functional microbial cores obtained a balanced AD process in both experimental phases, as shown by results of the GSTR performances at the steady state. Therefore, the whole microbial communities were able to adapt to H_2, as well as to the HDPE supports, thus, maintaining all the metabolic syntrophic relationships over the experimental time.

Author Contributions: Conceptualization, S.R., M.F., and A.S; methodology, G.L. and V.M.M.; investigation, G.L. and A.S.; data curation, G.L. and A.S.; writing—original draft preparation, G.L.;

writing—review and editing, G.L.,S.R., A.M.,G.M., and A.S.; visualization, S.R., G.M., and A.S.; supervision, M.F. and A.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

2.5 Abbreviations

AD	Anaerobic digestion
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
CW	Cheese whey
EF	Effluent
FISH	Fluorescent *in situ* hybridization
GSTR	Gas-stirred tank reactor
HDPE	High-density polyethylene
HRT	Hydraulic retention time
IM	Interstitial matrix
MER	Methane evolution rate
NGS	Next-generation sequencing
OLR	Organic loading rate
SCW	Second cheese whey
SMY	Specific methane yield
TS	Total solid
TVFA	Total volatile fatty acid
UP	Upgrading phase

VFA	Volatile fatty acid
VS	Volatile solid

2.6 References

Agneessens, L.M., Ottosen, L.D.M., Andersen, M., Berg Olesen, C., Feilberg, A., Kofoed, M.V.W., 2018. Parameters affecting acetate concentrations during *in-situ* biological hydrogen methanation. Bioresour. Technol. 258, 33–40.

Agneessens, L.M., Ottosen, L.D.M., Voigt, N.V., Nielsen, J.L., de Jonge, N., Fischer, C.H., Kofoed, M.V.W., 2017. *In-situ* biogas upgrading with pulse H_2 additions: The relevance of methanogen adaption and inorganic carbon level. Bioresour. Technol. 233, 256–263.

Ahmad, T., Aadil, R.M., Ahmed, H., Rahman, U. ur, Soares, B.C.V., Souza, S.L.Q., Pimentel, T.C., Scudino, H., Guimarães, J.T., Esmerino, E.A., Freitas, M.Q., Almada, R.B., Vendramel, S.M.R., Silva, M.C., Cruz, A.G., 2019. Treatment and utilization of dairy industrial waste: A review. Trends Food Sci. Technol. 88, 361–372.

Alfaro, N., Fdz-Polanco, M., Fdz-Polanco, F., Díaz, I., 2019. H_2 addition through a submerged membrane for *in-situ* biogas upgrading in the anaerobic digestion of sewage sludge. Bioresour. Technol. 280, 1–8.

Ali Shah, F., Mahmood, Q., Maroof Shah, M., Pervez, A., Ahmad Asad, S., 2014. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. Sci. World J. 2.

Amann, R., Binder, B., Olson, R., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Envir. Microbiol. 56, 1919–1925.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-olignucleotide probing of whole cells for determinitive, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. Biotechnol. Adv. 36, 452–466.

Angelidaki, I., Xie, L., Luo, G., Zhang, Y., Oechsner, H., Lemmer, A., Munoz, R., Kougias, P.G., 2019. Biogas upgrading: Current and emerging technologies, Biomass, Biofuels, Biochemicals: Biofuels: Alternative Feedstocks and Conversion Processes for the Production of Liquid and Gaseous Biofuels.

Aryal, N., Kvist, T., Ammam, F., Pant, D., Ottosen, L.D.M., 2018. An overview of microbial biogas enrichment. Bioresour. Technol. 264, 359–369.

Asunis, F., Gioannis, G. De, Dessì, P., Isipato, M., Lens, P.N.L., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2020. The dairy biorefinery : Integrating treatment processes for cheese whey valorisation. J. Environ. Manage. 276, 111240.

Awe, O.W., Zhao, Y., Nzihou, A., Minh, D.P., Lyczko, N., 2017. A Review of Biogas Utilisation, Purification and Upgrading Technologies. Waste and Biomass Valorization 8, 267–283.

Baek, G., Kim, Jaai, Kim, Jinsu, Lee, C., 2018. Role and potential of direct interspecies electron transfer in anaerobic digestion. Energies 11, 107–125.

Barra Caracciolo, A., Grenni, P., Cupo, C., 2005. *In situ* analysis of native microbial communities in complex samples with high particulate loads. FEMS Microbiol. 253, 55–58.

Bassani, I., Kougias, P.G., Angelidaki, I., 2016. *In-situ* biogas upgrading in thermophilic granular UASB reactor: key factors affecting the hydrogen mass transfer rate. Bioresour. Technol. 221, 485–491.

Bassani, I., Kougias, P.G., Treu, L., Angelidaki, I., 2015. Biogas Upgrading via Hydrogenotrophic Methanogenesis in Two-Stage Continuous Stirred Tank Reactors at Mesophilic and Thermophilic Conditions. Environ. Sci. Technol. 49, 12585–12593.

Bassani, I., Kougias, P.G., Treu, L., Porté, H., Campanaro, S., Angelidaki, I., 2017. Optimization of hydrogen dispersion in thermophilic up-flow reactors for *ex situ* biogas upgrading. Bioresour. Technol. 234, 310–319.

Braga Nan, L., Trably, E., Santa-Catalina, G., Bernet, N., Delgenès, J.P., Escudié, R., 2020. Biomethanation processes: New insights on the effect of a high H_2 partial pressure on microbial communities. Biotechnol. Biofuels 13, 1–18.

British Petroleum Report, 2018. 67 th Statistical Review of World Energy.

Burkhardt, M., Busch, G., 2013. Methanation of hydrogen and carbon dioxide. Appl. Energy 111, 74–79.

Campanaro, S., Treu, L., Kougias, P.G., De Francisci, D., Valle, G., Angelidaki, I., 2016. Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnol. Biofuels 9, 26.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat. Publ. Gr. 7, 335–336.

Carvalho, F., Prazeres, A.R., Rivas, J., 2013. Cheese whey wastewater: Characterization and treatment. Sci. Total Environ. 445–446, 385–396.

De Francisci, D., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2015. Microbial diversity and dynamicity of biogas reactors due to radical changes of feedstock composition. Bioresour. Technol. 176, 56–64.

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Rev. Environ. Sci. Biotechnol. 7, 173–190.

Demirel, B., Yenigun, O., Onay, T.T., 2005. Anaerobic treatment of dairy wastewaters: A review. Process Biochem. 40, 2583–2595.

Diamantis, V.I., Kapagiannidis, A.G., Ntougias, S., Tataki, V., Melidis, P., Aivasidis, A., 2014. Two-stage CSTR-UASB digestion enables superior and alkali addition-free cheese whey treatment. Biochem. Eng. J. 84, 45–52.

Díaz, I., Pérez, C., Alfaro, N., Fdz-Polanco, F., 2015. A feasibility study on the bioconversion of CO_2 and H_2 to biomethane by gas sparging through polymeric membranes. Bioresour. Technol. 185, 246–253.

Dinamarca, C., Bakke, R., 2012. Simultaneous hydrogen production and consumption in Anaerobic mixed culture fermentation. Int. J. Energy Environ. 3, 323–332.

Dinamarca, C., Gañán, M., Liu, J., Bakke, R., 2011. H_2 consumption by anaerobic non-methanogenic mixed cultures. Water Sci. Technol. 63, 1582–1589.

European Biogas Association, 2018. EBA Statistical Report 2018. Annu. Rep. 68.

Faisal, S., Salama, E.S., Hassan, S.H.A., Jeon, B.H., Li, X., 2020. Biomethane enhancement via plastic carriers in anaerobic co-digestion of agricultural wastes. Biomass Convers. Biorefinery.

Felchner-Zwirello, M., Winter, J., Gallert, C., 2013. Interspecies distances between propionic acid degraders and methanogens in syntrophic consortia for optimal hydrogen transfer. Appl. Microbiol. Biotechnol. 97, 9193–9205.

Fernández, C., Cuetos, M.J., Martínez, E.J., Gómez, X., 2015. Thermophilic anaerobic digestion of cheese whey: Coupling H 2 and CH 4 production. Biomass and Bioenergy 81, 55–62.

Ferraro, A., Massini, G., Mazzurco Miritana, V., Rosa, S., Signorini, A., Fabbricino, M., 2020. A novel enrichment approach for anaerobic digestion of lignocellulosic biomass: Process performance enhancement through an inoculum habitat selection. Bioresour. Technol. 313, 123703–123712.

Ferry, J.G., 2010. The chemical biology of methanogenesis. Planet. Space Sci. 58, 1775–1783.

Fontana, A., Campanaro, S., Treu, L., Kougias, P.G., Cappa, F., Morelli, L., Angelidaki, I., 2018a. Performance and genome-centric metagenomics of thermophilic single and two-stage anaerobic digesters treating cheese wastes. Water Res. 134, 181–191.

Fontana, A., Kougias, P.G., Treu, L., Kovalovszki, A., Valle, G., Cappa, F., Morelli, L., Angelidaki, I., Campanaro, S., 2018b. Microbial activity response to hydrogen injection in thermophilic anaerobic digesters revealed by genome-centric metatranscriptomics. Microbiome 6, 1–14.

Gagliano, M.C., Braguglia, C.M., Petruccioli, M., Rossetti, S., 2015. Ecology and biotechnological potential of the thermophilic fermentative Coprothermobacter spp. FEMS Microbiol. Ecol. 91, 1–12.

Ghaib, K., Ben-Fares, F.Z., 2018. Power-to-Methane: A state-of-the-art review. Renew. Sustain. Energy Rev. 81, 433–446.

Ghosh, S., Chowdhury, R., Bhattacharya, P., 2016. Mixed consortia in bioprocesses: role of microbial interactions. Appl. Microbiol. Biotechnol. 100, 4283–4295.

Goli, A., Shamiri, A., Khosroyar, S., Talaiekhozani, A., Sanaye, R., Azizi, K., 2019. A review on different aerobic and anaerobic treatment methods in dairy industry wastewater. J. Environ. Treat. Tech. 7, 113–141.

Gorrasi, S., Izzo, G., Massini, G., Signorini, A., Barghini, P., Fenice, M., 2014. From polluting seafood wastes to energy. production of hydrogen and methane from raw chitin material by a two-phase process. J. Environ. Prot. Ecol. 15, 526–536.

Götz, M., Lefebvre, J., Mörs, F., McDaniel Koch, A., Graf, F., Bajohr, S., Reimert, R., Kolb, T., 2016. Renewable Power-to-Gas: A technological and economic review. Renew. Energy 85, 1371–1390.

Guebitz, G.M., Bauer, A., Bochmann, G., Gronauer, A., Weiss, S., 2015. Biogas Science and Technology, Biogas Science and Technology.

Gujer, W., Zehnder, A.J.B., 1983. Conversion processes in anaerobic digestion. Water Sci. Technol. 15, 127–167.

Hattori, S., 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. 23, 118–127.

Ho, D., Jensen, P., Batstone, D., 2014. Effects of temperature and hydraulic retention time on acetotrophic pathways and performance in high-rate sludge digestion. Environ. Sci. Technol. 48, 6468–6476.

Hulshoff Pol, L.W., De Castro Lopes, S.I., Lettinga, G., Lens, P.N.L., 2004. Anaerobic sludge granulation. Water Res. 38, 1376–1389.

Ishii, S., Kosaka, T., Hotta, Y., Watanabe, K., 2006. Simulating the contribution of coaggregation to interspecies hydrogen fluxes in syntrophic methanogenic consortia. Appl. Environ. Microbiol. 72, 5093–5096.

Izzo, G., Rosa, S., Massing, G., Patriarca, C., Fenice, M., Fiocchetti, F., Marone, A., Varrone, C., Signorini, A., 2014. From hypertrophic lagoons to bioenergy production. J. Environ. Prot. Ecol. 15, 537–546.

Jabari, L., Gannoun, H., Cayol, J.L., Hamdi, M., Fauque, G., Ollivier, B., Fardeau, M.L., 2012. Characterization of defluviitalea saccharophila gen. nov., sp. nov., a thermophilic bacterium isolated from an upflow anaerobic filter treating abattoir wastewaters, and proposal of defluviitaleaceae fam. nov. Int. J. Syst. Evol. Microbiol. 62, 550–555.

Kapoor, R., Ghosh, P., Kumar, M., Vijay, V.K., 2019. Evaluation of biogas upgrading technologies and future perspectives: a review. Environ. Sci. Pollut. Res. 26, 11631–11661.

Karadag, D., Köroğlu, O.E., Ozkaya, B., Cakmakci, M., 2015. A review on anaerobic biofilm reactors for the treatment of dairy industry wastewater. Process Biochem. 50, 262–271.

Kato, S., Hashimoto, K., Watanabe, K., 2012. Microbial interspecies electron transfer via electric currents through conductive minerals. Proc. Natl. Acad. Sci. U. S. A. 109, 10042–10046.

Khemkhao, M., Nuntakumjorn, B., Techkarnjanaruk, S., Phalakornkule, C., 2011. Effect of chitosan on UASB treating POME during a transition from mesophilic to thermophilic conditions. Bioresour. Technol. 102, 4678–4681.

Koller, M., Gasser, I., Schmid, F., Berg, G., 2011. Linking ecology with economy: Insights into polyhydroxyalkanoate-producing microorganisms. Eng. Life Sci. 11, 222–237.

Kougias, P.G., Angelidaki, I., 2018. Biogas and its opportunities—A review. Front. Environ. Sci. Eng. 12, 1–12.

Kougias, P.G., Treu, L., Benavente, D.P., Boe, K., Campanaro, S., Angelidaki, I., 2017. *Ex-situ* biogas upgrading and enhancement in different reactor systems. Bioresour. Technol. 225, 429–437.

Krakat, N., Schmidt, S., Scherer, P., 2011. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. Bioresour. Technol. 102, 5692–5701.

Lane, N., Martin, W.F., Raven, J.A., Allen, J.F., 2013. Energy, genes and evolution: Introduction to an evolutionary synthesis. Philos. Trans. R. Soc. B Biol. Sci. 368, 1–5.

Lecker, B., Illi, L., Lemmer, A., Oechsner, H., 2017. Biological hydrogen methanation – A review. Bioresour. Technol. 245, 1220–1228.

Lembo, G., Massini, G., Mazzurco Miritana, V., Fenice, M., Felici, C., Liberatore, R., Signorini, A., 2016. Anaerobic digestion of ricotta cheese whey : effect of phase separation on methane production and microbial community structure, in: European Biomass Conference and Exhibition Proceedings, 2016 (24thEUBCE). ETA-Florence Renewable Energies, pagg. 599–607.

Lembo, G., Rosa, S., Miritana, V.M., Marone, A., Massini, G., Fenice, M., Signorini, A., 2021. Thermophilic anaerobic digestion of second cheese whey: Microbial community response to H_2 addition in a partially immobilized anaerobic hybrid reactor. Processes 9, 1–16.

Li, D., Ran, Y., Chen, L., Cao, Q., Li, Z., Liu, X., 2018. Instability diagnosis and syntrophic acetate oxidation during thermophilic digestion of vegetable waste. Water Res. 139, 263–271.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171–189.

Luo, G., Angelidaki, I., 2013a. Hollow fiber membrane based H_2 diffusion for efficient *in situ* biogas upgrading in an anaerobic reactor. Appl. Microbiol. Biotechnol. 97, 3739–3744.

Luo, G., Angelidaki, I., 2013b. Co-digestion of manure and whey for *in situ* biogas upgrading by the addition of H_2: Process performance and microbial insights. Appl. Microbiol. Biotechnol. 97, 1373–1381.

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., Angelidaki, I., 2012. Simultaneous hydrogen utilization and *in situ* biogas upgrading in an anaerobic reactor. Biotechnol. Bioeng. 109, 1088–1094.

Maegaard, K., Garcia-Robledo, E., Kofoed, M.V.W., Agneessens, L.M., de Jonge, N., Nielsen, J.L., Ottosen, L.D.M., Nielsen, L.P., Revsbech, N.P., 2019. Biogas upgrading with hydrogenotrophic methanogenic biofilms. Bioresour. Technol. 287, 121422.

Mainardis, M., Flaibani, S., Trigatti, M., Goi, D., 2019. Techno-economic feasibility of anaerobic digestion of cheese whey in small Italian dairies and effect of ultrasound pre-treatment on methane yield. J. Environ. Manage. 246, 557–563.

Martin, M.R., Fornero, J.J., Stark, R., Mets, L., Angenent, L.T., 2013. A single-culture bioprocess of methanothermobacter thermautotrophicus to upgrade digester biogas by CO_2-to-CH_4 conversion with H_2. Archaea 2013, 157529.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J.M., Schink, B., Rohlin, L., Gunsalus, R.P., 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Ann. N. Y. Acad. Sci. 1125, 58–72.

Mulat, D.G., Mosbæk, F., Ward, A.J., Polag, D., Greule, M., Keppler, F., Nielsen, J.L., Feilberg, A., 2017. Exogenous addition of H_2 for an *in situ* biogas upgrading through biological reduction of carbon dioxide into methane. Waste Manag. 68, 146–156.

Muñoz, R., Meier, L., Diaz, I., Jeison, D., 2015. A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Rev. Environ. Sci. Biotechnol. 14, 727–759.

Nakasaki, K., Nguyen, K.K., Ballesteros, F.C., Maekawa, T., Koyama, M., 2020. Characterizing the microbial community involved in anaerobic digestion of lipid-rich wastewater to produce methane gas. Anaerobe 61, 102082.

Narayanan, C.M., Narayan, V., 2019. Biological wastewater treatment and bioreactor design: A review. Sustain. Environ. Res. 1, 1–17.

Narihiro, T., Terada, T., Ohashi, A., Kamagata, Y., Nakamura, K., Sekiguchi, Y., 2012. Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment systems by sequence-specific rRNA cleavage method. Water Res. 46, 2167–2175.

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739.

O'Flaherty, V., Collins, G., Mahony, T., 2006. The microbiology and biochemistry of anaerobic bioreactors with relevance to domestic sewage treatment. Rev. Environ. Sci. Biotechnol. 5, 39–55.

Patterson, T., Esteves, S., Dinsdale, R., Guwy, A., Maddy, J., 2013. Life cycle assessment of biohydrogen and biomethane production and utilisation as a vehicle fuel. Bioresour. Technol. 131, 235–245.

Pavan, M.E., Pavan, E.E., Glaeser, S.P., Etchebehere, C., Kämpfer, P., Pettinari, M.J., López, N.I., 2018. Proposal for a new classification of a deep branching bacterial phylogenetic lineage: Transfer of Coprothermobacter proteolyticus and Coprothermobacter platensis to Coprothermobacteraceae fam. nov., within Coprothermobacterales ord. nov., Coprothermobacte. Int. J. Syst. Evol. Microbiol. 68, 1627–1632.

Pervin, H.M., Dennis, P.G., Lim, H.J., Tyson, G.W., Batstone, D.J., Bond, P.L., 2013. Drivers of microbial community composition in mesophilic and thermophilic temperature-phased anaerobic digestion pre-treatment reactors. Water Res. 47, 7098–7108.

Pohland, F.G., Ghosh, S., 1971. Developments in anaerobic stabilization of organic wastes - the two-phase concept. Environ. Lett. 1, 255–266.

Porté, H., Kougias, P.G., Alfaro, N., Treu, L., Campanaro, S., Angelidaki, I., 2019. Process performance and microbial community structure in thermophilic trickling biofilter reactors for biogas upgrading. Sci. Total Environ. 655, 529–538.

R.I. Amann, K.H. Ludwig, K.H.S., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Mol. Biol. Rev. 24, 143–169.

Rachbauer, L., Voitl, G., Bochmann, G., Fuchs, W., 2016. Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. Appl. Energy 180, 483–490.

Rajendran, K., Mahapatra, D., Venkatraman, A.V., Muthuswamy, S., Pugazhendhi, A., 2020. Advancing anaerobic digestion through two-stage processes: Current developments and future trends. Renew. Sustain. Energy Rev. 123, 109746.

Ramasamy, E.., Abbassi, S.., 2000. Energy recovery from dairy waste-waters: impacts of biofilm support systems on anaerobic CST reactors. Appl. Energy 65, 91–98.

Ramsay, I.R., Pullammanappallil, P.C., 2001. Protein degradation during anaerobic wastewater treatment: Derivation of stoichiometry. Biodegradation 12, 247–257.

Regueira, A., González-Cabaleiro, R., Ofiţeru, I.D., Rodríguez, J., Lema, J.M., 2018. Electron bifurcation mechanism and homoacetogenesis explain products yields in mixed culture anaerobic fermentations. Water Res. 141, 349–356.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3, 700–714.

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy 38, 13172–13191.

Sarker, S., Lamb, J.J., Hjelme, D.R., Lien, K.M., 2018. Overview of recent progress towards *in-situ* biogas upgradation techniques. Fuel 226, 686–697.

Schink, B., 2006. Syntrophic associations in methanogenic degradation. Prog. Mol. Subcell. Biol. 41, 1–19.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61, 262–280.

Shen, L., Zhao, Q., Wu, X., Li, X., Li, Q., Wang, Y., 2016. Interspecies electron transfer in syntrophic methanogenic consortia: From cultures to bioreactors. Renew. Sustain. Energy Rev. 54, 1358–1367.

Stams, A.J.M., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7, 568–577.

Sun, Q., Li, H., Yan, J., Liu, L., Yu, Z., Yu, X., 2015. Selection of appropriate biogas upgrading technology-a review of biogas cleaning, upgrading and utilisation. Renew. Sustain. Energy Rev. 51, 521–532.

Tandishabo, K., Nakamura, K., Umetsu, K., Takamizawa, K., 2012. Distribution and role of Coprothermobacter spp. in anaerobic digesters. J. Biosci. Bioeng. 114, 518–520.

Treu, L., Tsapekos, P., Peprah, M., Campanaro, S., Giacomini, A., Corich, V., Kougias, P.G., Angelidaki, I., 2019. Microbial profiling during anaerobic digestion of cheese whey in reactors operated at different conditions. Bioresour. Technol. 275, 375–385.

Turner, J., Sverdrup, G., Mann, M.K., Maness, P.C., Kroposki, B., Ghirardi, M., Evans, R.J., Blake, D., 2008. Renewable hydrogen production. Int. J. Energy Res. 32, 379–407.

Vasmara, C., Marchetti, R., 2017. Initial pH influences in-batch hydrogen production from scotta permeate. Int. J. Hydrogen Energy 42, 14400–14408.

Wang, W., Xie, L., Luo, G., Zhou, Q., Angelidaki, I., 2013. Performance and microbial community analysis of the anaerobic reactor with coke oven gas biomethanation and *in situ* biogas upgrading. Bioresour. Technol. 146, 234–239.

Xia, A., Cheng, J., Murphy, J.D., 2016. Innovation in biological production and upgrading of methane and hydrogen for use as gaseous transport biofuel. Biotechnol. Adv. 34, 451–472.

Xia, Y., Wang, Y., Fang, H.H.P., Jin, T., Zhong, H., Zhang, T., 2014. Thermophilic microbial cellulose decomposition and methanogenesis pathways recharacterized by metatranscriptomic and metagenomic analysis. Sci. Rep. 4, 1–9.

Yang, K., Yu, Y., Hwang, S., 2003. Selective optimization in thermophilic acidogenesis of cheese-whey wastewater to acetic and butyric acids : partial acidification and methanation 37, 2467–2477.

Yue, Z., Chen, R., Yang, F., MacLellan, J., Marsh, T., Liu, Y., Liao, W., 2013. Effects of dairy manure and corn stover co-digestion on anaerobic microbes and corresponding digestion performance. Bioresour. Technol. 128, 65–71.

Zabranska, J., Pokorna, D., 2018. Bioconversion of carbon dioxide to methane using hydrogen and hydrogenotrophic methanogens. Biotechnol. Adv. 36, 707–720.

Zhang, L., Kuroki, A., Tong, Y.W., 2020. A Mini-Review on *In situ* Biogas Upgrading Technologies via Enhanced Hydrogenotrophic Methanogenesis to Improve the Quality of Biogas From Anaerobic Digesters. Front. Energy Res. 8, 1–7.

Zhu, X., Chen, L., Chen, Y., Cao, Q., Liu, X., Li, D., 2019. Differences of methanogenesis between mesophilic and thermophilic *in situ* biogas-upgrading systems by hydrogen addition. J. Ind. Microbiol. Biotechnol. 46, 1569–1581.

Zotta, T., Solieri, L., Iacumin, L., Picozzi, C., Gullo, M., 2020. Valorization of cheese whey using microbial fermentations. Appl. Microbiol. Biotechnol. 104, 2749–2764.

3 *In situ* biogas upgrading in a in a partially immobilized anaerobic hybrid reactor

3.1 Introduction

Anaerobic digestion (AD) is a biological process which, in absence of oxygen, degrades organic matter to a biogas mainly composed of CH_4 (~50-60%) and CO_2 (~50-40%). AD process is widely used as sustainable technology for biogas production (Gorrasi et al., 2014).The most common utilization of biogas is the electricity production combined with the utilization of the excess heat by a co-generation engine (CHP units). Alternatively, biogas can be upgraded to natural gas quality (biomethane) and used as vehicle fuel or injected into the existing natural gas grid too. (Bassani et al., 2017)(Ghaib e Ben-Fares, 2018; Kapoor et al., 2019; Kougias e Angelidaki, 2018). Nowadays physical biogas upgrading methods using organic solvent absorption, water scrubbing, pressure swing adsorption, cryogenic or membrane separation, are already commercially used in the biogas industry. However these technologies increase biogas production costs by 20-72% due to high investment, electricity demand or chemical/water requirement. An attractive and cost effective method to increase the CH_4 content in biogas is based on the biological conversion of CO_2 and H_2 to CH_4, and it is performed by anaerobic microorganisms. Currently two main process approaches are used for the biological methanation: the *in situ* methanation, which consists of the direct injection of H_2 into the anaerobic digester, using internally produced CO_2 and the *ex situ* methanation, realized into a separate reactor containing enriched cultures of hydrogenotrophic methanogens where H_2 and CO_2 or H_2 and biogas are injected. *In situ* biogas upgrading option requires less additional investment costs and it can be easily integrated with the already existing anaerobic reactor at biogas plants (Zabranska and Pokorna, 2018; Alfaro et al., 2019), among which the continuously stirring reactor (CSTR) configuration is the most popular employed for anaerobic treatment of industrial, domestic and municipal wastes (Narayanan e Narayan, 2019). Several studies suggest that the H_2 diffusion in the liquid phase is the limiting factor of the process since the low H_2 solubility make it not bioavailable for microorganisms (Alfaro et al., 2019; Bassani et al., 2016; Díaz et al., 2015; Martin et al., 2013). In order to overcome this limit, several *in situ* strategies for CSTR reactor configuration have been proposed, mainly introducing different H_2 injection devices. In small-scale reactor (working volume $\leq$ 3.5L), the H_2 was introduced by column or ceramic diffusers (Luo et al., 2012; Luo e Angelidaki, 2013b), hollow fiber membrane (HFM) and headspace injection (Agneessens et al., 2017), all using different stirring speeds. In these studies the CH_4 increase due to the conversion of the H_2 added, was expressed as methane evolution rate (MER) and it ranged from 0.08 to 0.39 L L_r^{-1} d^{-1} with a corresponding concentration of CH_4 included in the range 53%-96%. Although with the use of HFM the best results were

obtained, the connected costs have to be considered for full-scale applications (Angelidaki et al., 2018). Moreover, further limits are related to the need of injecting the gas into the thin pores using high pressure as well as to the biofilm formation which can damage the permeability and the lifespan of the membrane itself. Alfaro et al. (2019) proposed biogas recirculation in a 20L CSTR implemented with a HFM. Although the results showed the positive effect of biogas recirculation, the use of a membrane area/reactor volume ratio twenty-four times lower than that experienced in a previous study, lead to a decrease of MER from 0.38 to 0.16 $LCH_4\ L_r^{-1}\ d^{-1}$ and of CH_4 content in the biogas from 96% to 73.1 % (Alfaro et al., 2019; Luo e Angelidaki, 2013b).

Studies on *ex situ* biomethanation, showed that contact area between liquid and gas flow is markedly increased when filling the reactor with packing material (Bassani et al., 2017; Burkhardt e Busch, 2013; Rachbauer et al., 2016). Recently, Kougias et al. 2020 investigated both the effect of packing material and biogas recirculation rate on CO_2 fixation in up-flow reactors fed with exogenous H_2. The results showed that in a reactor filled with Raschig rings both CO_2 and H_2 utilization efficiency were increased and that intense biogas recirculation rate is a prerequisite for achieving high CH_4 purity. In addition microbial diversity composition of the effluent showed a higher relative abundance of *Methanothermobacter* specie, a hydrogenotrophic methanogenes, working in syntrophic relation with homoacetogenes *Pseudomonas* sp.

The exogenous H_2 injection into the AD digester, can also affect the pH and at the same time the composition and the activity of syntrophic microbial community. The increase of pH at value higher than 8.5 due to CO_2 removal, can cause inhibition of methanogenesis. On the other hand, an increase of H_2 partial pressure can affect the metabolic pathways of acetogenesis phase causing a buildup of VFAs and a decrease of pH toward inhibitory values of methanogenesis. In addition, the conversion of H_2 and CO_2 to acetate by homoacetogenesis, is more favorable under high H_2 partial pressure and alkaline pH than hydrogenotrophic pathway (Schink, 1997). Thus homoacetogenes can outcompete with hydrogenotrophic methanogens for H_2 utilization, and they can potentially inhibit AD process if they didn't interact syntrophically with acetoclastic methanogens.

Our previous investigation on microbial community during *in situ* biomethanation, reported the effects of H_2 addition on microbial community abundance and structure, in a partially immobilized GSTR reactor (Gas Stirred Tank Ractor) by polyethylene carriers. The results showed an increase of the microbial methanogenic communities' abundance in both effluent (134%) and interstitial matrix inside the immobilized area (46%). On the contrary, abundances of bacterial communities decreased. In addition, both Bacteria and Archaea communities structure were affected by the H_2

addition according to a site-specific development: they both colonized the effluent and the interstitial matrix inside the immobilized area with peculiar microbial families, thus creating specific microbial cores. Although this study was not attempted to optimize biomethanation, the results showed a 16% increase in methane production rate at a biogas recirculation rate of 118 L L_r^{-1} d^{-1}.

The aim of this study was to investigate the effects both of H_2 addition and different biogas recirculation rates on *in situ* H_2 upgrading efficiency, organic matter removal and microbial community structure in a hybrid gas-stirred tank reactor (GSTR) where about one third of the reactor working volume is filled with polymeric supports (high-density polyethylene, HDPE). The reactor was continuously fed with second cheese whey (SCW), a waste stream of dairy industry and the AD process was conducted at thermophilic condition (55°C).

3.2 Materials and Methods

3.2.1 Inoculum and Substrate

Thermophilic digested sludge was used as inoculum for our previous activities (Lembo et al., 2021). SCW used as substrate was periodically collected from a small dairy factory located in Rome, Italy. The wastewater was stored at 20 °C and thawed before use. Undiluted SCW was fed into the GSTR reactor. Due to the fluctuations of the residual organic load derived from the production process, slight variations in its composition were observed. The main physical and chemical characteristics of the SCW are presented in Table 1.

Table 1. Main characteristics of second cheese whey (SCW) used in this study.

Parameters	Range
pH	5.9-6.2
Lactose(gL^{-1})	40-60
Total Solids (TS) (gL^{-1})	47-64
Volatile Solids (VS) (gL^{-1})	40-54
COD (gL^{-1})	45-70
Proteins (gL^{-1})	0.45-0.90
$NH4^{+}$(gL^{-1})	0.10-2.5
Total Volatile fatty acids (TVFAs) (gL^{-1})	1.5-2.5

3.2.2 Reactors set up and operation

The experiment was carried out in a hybrid Gas Stirred Tank Reactor (GSTR) with working volume of 49L at thermophilic temperature (55 ± 1°C) and atmospheric pressure. The reactor configuration was described in a previously study (Lembo et al., 2021). Undiluted RCW was used as fed in continuously mode from the bottom using a peristaltic pump. The hydraulic retention time (HRT) was controlled at 30 days and the organic loading rate (OLR) was, on average, within a range of 1.16 ± 0.05 gVS L^{-1} d^{-1} and 1.28 ± 0.08 gVS L^{-1} d^{-1}.

Initially, a biogas recirculation flow rate of 118 L L^{-1} d^{-1} was applied and no biogas upgrading occurred. This period is considered as control, and it is indicated as AD. When a stable AD process was achieved, pure H_2 (≥99%) obtained from an electrolyzer (DBS, model PG-H_2 100), was continuously injected into the reactor from the bottom, by of biogas recirculation line. The starting H_2 flow rate was 1.18 L_{H2} L_r^{-1} day^{-1} and gas recirculation flow rate was maintained at. 1.18 L L_r^{-1} d^{-1} This experimental phase is denoted as Upgrading 1 (UP1). Thereafter, H_2 flow rate was increased to 1.47 L_{H2} L_r^{-1} day^{-1} and gas recirculation flow was maintained stable. This experimental phase is indicated as Upgrading 2 (UP2) Both UP1 and UP2 lasted for 30 days. Afterwards it was kept stable the H_2 flow rate at to 1.47 L_{H2} L_r^{-1} day^{-1} and gas recirculation flow was increased initially to 176 L L_r^{-1} d^{-1} , this experimental phase is denoted as Upgrading 3 (UP3) and subsequently gas recirculation flow was increased was increase up to 235 L L_r^{-1} d^{-1} this experimental phase is denoted as Upgrading 4 (UP4). Table 2 report a detailed scheme of the different experimental phases

Table 2. Scheme of the different experimental phases

Parameters	AD	UP1	UP2	UP3	UP4
OLR (g VS L_r^{-1} d^{-1})	1.20 ± 0.02	1.16 ± 0.08	1.16 ± 0.05	1.26 ± 0.09	1.28 ± 0.08
H_{2In} Flow rate (L_{H2} L_r^{-1} d^{-1})	/	1.18 ± 0.01	1.47 ± 0.01	1.47 ± 0.01	1.47 ± 0.01
Biogas recirculation rate (L L_r^{-1} d^{-1})	118 ± 5	118 ± 5	118 ± 5	176 ± 5	235 ± 5

3.2.3 Analytical Methods

H_2, CH_4, CO_2, N_2, and O_2 percentages in biogas were analyzed by online Micro Gas Chromatograph Varian (GC4900). Two columns of 10 m were used. The first, MS 5A, had a stationary phase with molecular sieves capable of separating the permanent gases of low molecular

weight, H_2, CH_4, N_2, and O_2. The second column was a Poraplot U, used as a stationary phase divinyl benzene able to separate CO_2. Argon was used as the carrier gas. The biogas and H_2 flow rates were monitored by digital online flow meters (EL-Flow select series, Bronkhorst High-Tech B.V, Ruurlo Netherland).The flows of the different gases were calculated on the basis of the percentage compositions of the individual gas present in the biogas. Volatile fatty acids (VFAs), lactic acid, alcohols, and sugars were analyzed by a HPLC Thermo Spectrasystem, equipped with a UV detector (λ = 210 nm)and a refraction index detector, using the isocratic method of analysis at 75 ◦C with Column Rezex ROA-Organic Acid H+ (8%), size 300 × 7.8 mm Phenomenex, USA.

3.2.4 Calculation

Performance and efficiency of the methanation process were expressed as the methane evolution rate (MER), i.e, the rate of CH_4 production from the injected H_2, and H_2 conversion efficiency (ηH_2). The MER (Eq. 1) expresses the increase in the specific CH_4 production rate ($L_{CH4}\ L_r^{-1}d^{-1}$) caused by H_2 injection as compared with the CH_4 production rate in the control condition. It is calculated as follows (Equation (1):

$$MER = CH_{4UPs} - CH_{4AD} \quad (1)$$

where UPs are the upgrading periods (with H_2 addition) and AD is the control period (with- out H_2) The H_2 gas-liquid mass transfer rate, namely rt ($L_{H2}L_r^{-1}d^{-1}$), and the efficiency of H_2 utilization, namely ηH_2 (%), were calculated according to Equations (2) and (3), respectively:

$$Rt = H_2 \text{ in flow rate} - H_2 \text{ in output gas} \quad (2)$$

$$\eta H2 = \frac{\text{H2 in flow rate} - \text{H2 in output gas}}{\text{H2 in flow rate}} \text{ X } 100 \quad (3)$$

Where H_2 in flow rate and H_2 in output gas are the volumetric H_2 flows entering and leaving the reactor. It was assumed that all the H_2 transferred to the liquid phase was ultimately converted to CH_4 or used for other microbial metabolic pathways or employed for microbial growth (Alfaro et al., 2019; Díaz et al., 2015) .

The H_2 rate converted to biomethane ($L_{CH4}\ L_r^{-1}\ d^{-1}$) was calculated according to Equation (4):

$$\text{Rate to biomethane} = 4 \times (CH_4 \text{ in UP output gas} - CH_4 \text{ in AD output gas}) \quad (4)$$

where 4 is the stoichiometric coefficient according to Equation (4) $H_2 + CO_2 = CH_4$, CH_4 in UP output gas ($L_{CH4}\ L_r^{-1}\ d^{-1}$) is the rate of CH_4 produced in UP experimental phases, and CH_4 in AD output gas ($L_{CH4}\ L_r^{-1}\ d^{-1}$) is the rate of CH_4 produced in AD phase.

3.2.5 Illumina Sequencing

Samples for the microbial community analysis were collected at the end of AD and UP1 and UP4 phases and stored at -20°C until use. Genomic DNA was extracted as reported in Lembo et al. (Lembo et al., 2021) Library preparation was performed utilizing a 460bp amplicon corresponding to the hypervariable V3-V4 region of 16S rRNA using universal primers (S-D-Bact-0341F and S-D-Bact-0785R for bacteria and archaea domains). Sequencing was performed at the Department of Agricultural Sciences, University of Naples Federico II (Portici, Italy) using the Illumina MiSeq platform. From the generated reads, demultiplexing, quality filtering, trimming, merging, and operative taxonomical units (OTUs) picking were performed using QIIME pipeline (Caporaso et al., 2010). The taxonomical assignment was performed with the Greengenes database at a 97% similarity.

3.3 Results and discussion

3.3.1 Reactor performance

Process performance data at the steady state are summarized in Table 3. Figure 1 shows daily time-courses of biogas H_2, CH_4, CO_2 flow rates of all experimental phases.

Table 3. Process at the end of the all experimental phases.

Parameters	AD	UP1	UP2	UP3	UP4
OLR (g VS L_R^{-1} d^{-1})	1.19 ± 0.02	1.15 ± 0.08	1.15 ± 0.05	1.25 ± 0.09	1.28 ± 0.08
$H2_{in}$ Flow rate (L L_R^{-1} d^{-1})	/	1.18 ± 0.01	1.47 ± 0.01	1.47 ± 0.01	1.47 ± 0.01
Biogas recirculation rate (L L_r^{-1} d^{-1})	118 ± 5	118 ± 5	118 ± 5	176 ± 5	235 ± 5
Biogas production rate (L L_r^{-1} d^{-1})	0.61 ± 0.02	0.93 ± 0.02	0.94 ± 0.02	0.87 ± 0.03	0.80 ± 0.03
H2 %	/	31.1 ± 0.71	33.0 ± 0.21	27.0 ± 0.20	20.5 ± 0.13
CH4%	51.5 ± 0.40	55.8 ± 0.51	60. ± 0.83	67.8± 0.91	75.2 ± 0.53
CO2%	48.5 ± 0.40	13.10 ± 0.65	7.0 ± 0.58	5.22 ± 0.89	4.34 ± 0.48
H2 Flow rate (L Lr^{-1} d^{-1})	/	0.29 ± 0.01	0.31 ± 0.01	0.24 ± 0.01	0.16 ± 0.01
CH4 Flow rate (L Lr^{-1} d^{-1})	0.32 ± 0.01	0.52 ± 0.01	0.57 ± 0.03	0.59 ± 0.02	0.60 ± 0.02
rt (L L_R^{-1} d^{-1})	/	0.89 ± 0.01	1.14 ± 0.01	1.22 ± 0.01	1.28 ± 0.01
ηH2 (%)	/	75	79	84	89
MER ($L_{CH4}L_r^{-1}d^{-1}$)	ND	0.20	0.25	0.28	0.29
Lactose (mg/L)	224 ± 13	168 ± 12	148 ± 13	159 ± 6.85	172 ± 10
Acetic Acid (mg/L)	33 ± 1.8	32 ± 1.5	41 ± 7	159 ± 15	358 ± 10
pH	7.10 ± 0.14	7.81 ± 0.20	8.12 ± 0.0.18	8.20 ± 0.15	8.24 ± 0.10
Effluent VS (g/l)	4.78 ± 0.15	4.76 ± 0.12	4.81 ± 0.14	4.96 ± 0.21	4.85 ± 0.45

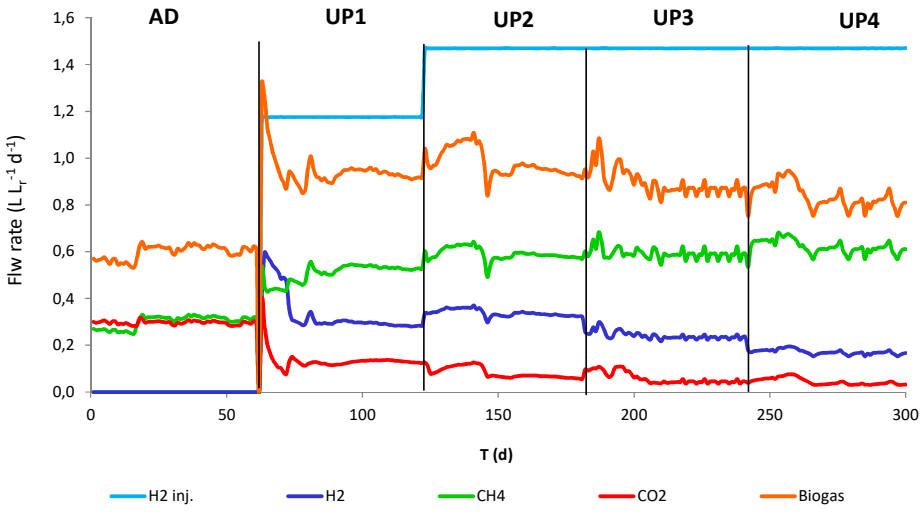

Figure 1. Daily time-courses of biogas H_2, CH_4, CO_2 flow rates of all experimental phases.

During AD period, a stable methane production rate of 0.32 ± 0.01 $L_{CH4}\,L_r^{-1}d^{-1}$ was achieved after 17 days. The pH of the reactor was around 7.10 ± 0.14 indicating a strong buffer capacity of the system despite any pH control was applied. The average content of CH_4 in the biogas was equal to 51.5 ± 0.40 % and the average SMY was equal to 0.270 ± 0.05 $L_{CH4}\,g_{VS}^{-1}$. The substrate was almost completely consumed; indeed, in the reactor effluent, lactose was detected in low concentration (224 ± 13 g L^{-1}) indicating an efficient performance of the AD phase. Acetic acid was the unique VFA detected in the effluent during AD process, with the concentration of 32.8 ± 1.8 g L^{-1}. After AD phase, UP1 started at day 60 by adding H_2 at the injection rate of 1.18 $L_{H2}\,L_r^{-1}\,d^{-1}$ into the reactor. The biogas recirculation rate was maintained at 118 L $L_r^{-1}\,d^{-1,}$ as in the previous phase. During the steady state of UP1, (80 to 120 days), the CH_4 production rate was 0.52 ± 0.01 $L_{CH4}\,L_r^{-1}\,d^{-1}$ which represented 65% of increase compared to AD phase but methane concentration in the biogas was only 55.8%± 0.5. The efficiency value of H_2 utilization (ηH_2) was 75%. Taking into account the stoichiometric injection of H_2, relatively high concentration of H_2 (31.10 ± 1.70) was detected in the output gas, and this was probably due to a limitation of GSTR configuration to obtain a complete H_2 gas-liquid mass transfer. In the following UP2 phase (121 to 180 days), a higher H_2 injecting rate was applied (1.47 $L_{H2}\,L_r^{-1}\,d^{-1}$), by keeping constant the biogas recirculation rate (i.e. 118 L $L_r^{-1}\,d^{-1}$). During the steady state of UP2 (reached after day 22) the CH_4 flow rate was 0.57 ± 0.02 $L_{CH4}\,L_r^{-1}\,d^{-1}$. The H_2 consumption rate (rt) increased from 0.89 observed in UP1 phase to 1.16 $L_{H2}\,L_r^{-1}\,d^{-1}$ and the percentage of CH_4 in the biogas increased up to 60 %. MER value was equal to 0.25 $L_{CH4}\,L_r^{-1}\,d^{-1}$and CO_2 flow rate in output gas was 54% lower than in UP1. At day 180, the gas recirculation rate was increased to 176 L $L_r^{-1}\,d^{-1}$, marking the beginning of UP3 phase (180 to 240 days) of the experiment. A further improvement of the H_2 mass transfer in UP3 period was observed (i.e. the rt was equal to 1.23 $L_{H2}\,L_r^{-1}\,d^{-1}$**)**, which corresponded to the 84% of increase of the efficiency of H_2 utilization. At the same time the CH_4 concentration in the biogas reached the value of 67.8%. A slightly increase in the MER value was also observed (0.28 $L_{CH4}\,L_r^{-1}\,d^{-1}$) compared to UP2 (0.25 $L_{CH4}\,L_r^{-1}\,d^{-1}$). On day 240, gas recirculation rate was increased to 235 L $L_r^{-1}\,d^{-1}$ with the purpose of raising ηH_2. In this stage, the average ηH_2 increase was 89, and the CH_4 concentration increased to 75.2% corresponding to 46% of increase as compared to AD. H_2 content dropped from 27% observed in UP3 to 20.5%. As a major result the CO_2 concentration was equal to 4.3%, which represented a decrease of 91% compared the concentration observed in AD phase (48%). As previously reported by other authors (Alfaro et al., 2019); (Kougias et al., 2020), we observed a positive effect of the increase of biogas recirculation rate on biogas upgrading. The process performances of biogas upgrading obtained in our study is comparable to the ones obtained using a reactor configuration (20 L of working volume) characterised by the biogas recirculation

and a hollow fiber membrane module.(Alfaro et al., 2019). To the best of our knowledge, the study of Alfaro et al. (2019) is the only one which uses biogas recirculation to increase biogas upgrading efficiency in *in situ* biomethanation process. At the end of the experimentation, the authors obtained a biogas with CH_4, H_2 and CO_2 content of 73% ± 3.4, 7.2% ± 2.4 and 19.7% ± 3 respectively, by applying a biogas recirculation rate of 202 L L_r^{-1} d^{-1}. Moreover a MER value of 0.16 L_{CH4} L_r^{-1} d^{-1} with an efficiency value of H_2 utilization of 94% was obtained. Comparatively, in the present study, higher MER value (0.29 L_{CH4} L_r^{-1} d^{-1}) was reached but the H_2 utilization efficiency was lower (89%). In the current study CH_4 concentration in the biogas reached a slightly higher value (75% ± 0.5), while higher concentration of hydrogen (20.5%) with a much lower CO_2 concentration (4.3%) in the biogas were observed. Similar low values of CO_2 contents were obtained by Luo and Angelidaki (2013) and Voelklein et al. (2019) i.e. 6.6% and 5.1% respectively. In both studies incomplete biogas upgrading was observed, whit CH_4 content in the biogas of 75% and 60.3% and whit H_2 content in the biogas of 18.4% and 34.6%, respectively. In order to enhance contact between the injected H_2 and the microbial community, these studies utilized specific operation conditions: the use of ceramic diffuser on the bottom of the reactor as gas diffusing system, the insertion both of stirring speed of 150 rpm and of biogas recirculation of 4 L/min.

In the present study, we adopted the use of immobilizing particles in GSTR configuration. Then, we assume that the immobilized area in GSTR configuration may have played a significant role in extending gas-liquid contact as a H_2 injection method. Further research are needed to elucidate the exact role of the immobilized particles in the gas-liquid mass transfer.

3.3.2 pH, VFAs evolution and organic matter removal

A main challenge that the biogas upgrading technology faces is related with the increment of pH level to values above 8.5, attributed to the removal of bicarbonate and leading also to inhibition of methanogenesis (Angelidaki et al., 2018). On the contrary, an increase of H_2 partial pressure due to the addition of H_2 can cause VFA and/or acetic acid to accumulate leading to acidification and reactor failure. During the experimental phases, it can be observed a major increase of pH from AD (7.10 ± 0.14) to UP1 phase (7.81 ± 0.20) and then a gradual increase to 8.24 ± 0.10 in UP4. Acetate was the unique VFA detected in all UP phases with an increasing concentration from UP2 to UP4. Similarly, acetate accumulation was reported in previous studies (Luo and Angelidaki, 2013b; Mulat et al., 2017; Agneessens et al., 2017; Kougias et al., 2020). However, since no other organic acids are present in the effluent suggests that the acetogenic phase of the AD process was not inhibited.

The injected H_2 may be converted to CH_4 by hydrogenotrophic methanogens but it may also be utilized by homoacetogens to produce acetate. On the other hand, acetate can be readily converted to CH_4 by acetoclastic methanogens. Then, the ongoing increase of acetate was a result of a stimulated homoacetogenesis pathway and a decreased activity of acetoclastic methanogens.

Finally, according to the results shown in Table 1, the H_2 addition and the biogas recirculation did not influence the VS concentration in the effluent.

3.3.3 Microbial communities

The microbial communities of liquid samples collected at the end of each experimental phase (AD,UP1,UP2, UP3 and UP4) as well as of the thermophilic sludge used as inoculum were analysed by Illumina-based 16S sequencing. The taxonomic assignment of the sequences at the different taxonomic levels decreased from the phyla (99%) toward the species (25%) levels, showing 97% (families) and 46% (genera) of representativeness. Although the gene amplicon sequencing approach is not appropriate to provide 16S rRNA gene sequences of sufficient length, it is possible to hypothesize that the low assignment at genera and species levels may suggest the presence in the microbioma of numerous unexplored or undescribed taxa. The results reported are focused on most abundant members of the microbial community having relative abundance ≥ 1.0%.

3.3.4 Bacteria communities

Bacterial population covered on average 99%-96% of the whole microbial community with the remaining 1%-4% being classified as *Archaea*.

The relative abundance of Bacteria communities at phylum level is shown in Figure 2.

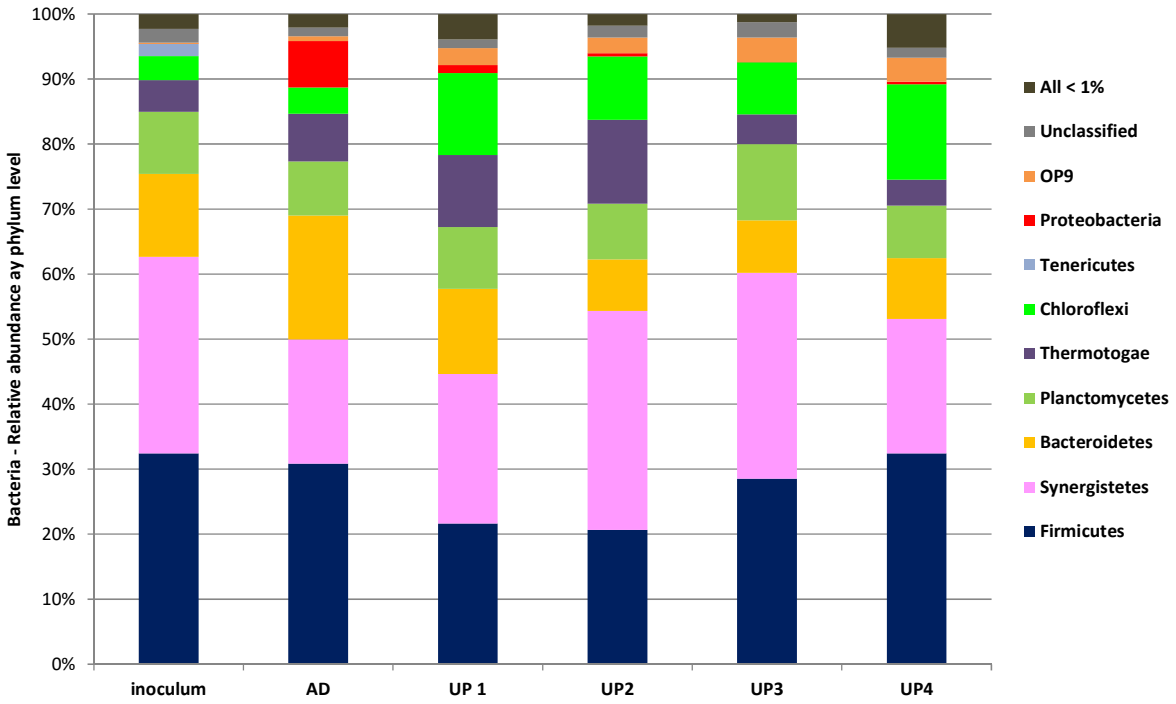

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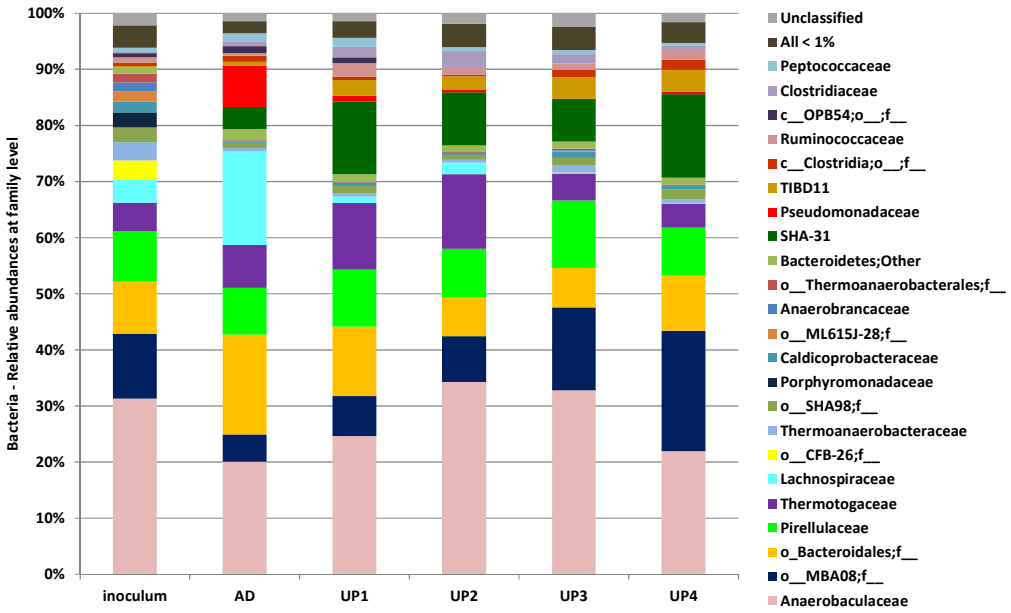
Figure 2. Relative abundances of the bacteria community at phylum level in the sludge used as inoculum, at the end of AD and UP1 and UP4 phases Communities with a relative abundance ≥1% in at least one sample are reported

In the thermophilic sludge used as inoculum *Firmicutes* and *Synergistetes* phyla with relative abundances of 33% and 31%, respectively, accounted for more than half of the whole microbial communities. Representatives of *Bacteroidete*s and *Planctomycetes* phyla were also abundant representing 13% and 10%, respectively. Minor representative phyla were identified in *Thermotogae* (5%) and *Chloroflexi* (4%) and in *Tenericutes (*2%) to a small extent. The microbial composition identified in the inoculum remained quite stable in the sample collected at the end of AD phase. The most relevant difference with the inoculum community was identified in the presence of *Proteobacteria* (7%) and *OP9* (1%) phyla. Moreover, *Firmicutes* (31%), *Sinergistetes* (19%) and *Bacteroidetes* (19%) phyla still represented a considerable fraction of the whole microbial communities, while members of *Planctomycetes* (8%), *Thermotogae* (7%) and *Chloroflexi* (4%) remained stable and members of *Tenericutes* completely disappeared.

Similarly, the relative abundances of microbial communities at the end of UP1, UP2, UP3 and UP4 experimental phases remained stable for members of *Firmicutes* (22%, 21%, 28% and 32%, respectively), *Sinergistetes* (23%, 34%, 31% and 21%, respectively), *Bacteroidetes* (13%, 8%, 8% and 9%, respectively) and *Planctomycetes* (10%, 9%, 12% and 8%, respectively), and a significant increase of members belonging to *Chloroflexi* phylum in UP1 (13%), UP2 (10%), UP3 (8%) and UP4 (15%) was also observed. Members of OP9 slightly increased (UP1=3%, UP2=2%, UP3=4% and UP4= 4%); to the opposite, members of *Proteobacteria* (7%) significantly decreased in UP1 and UP2 (1%) and they completely disappeared in UP3 and UP4.

The characterisation at phylum level highlighted as the whole microbial community of the sludge used as inoculum was already acclimatised to the thermophilic AD process treating SCW at the same operational HRT of 30 days. In addition, its composition was maintained quite stable throughout the AD, and all UP experimental phases. This behaviour can explain the stable performance of the AD process in our GSTR. A more detailed characterisation inside the microbial structure is obtained by the classification at family level (Figure 3).



Index	Inoculum	AD	UP1	UP2	UP3	UP4
H'	2.51	2.37	2.37	2.20	2.26	2.26
E	0.75	0.74	0.75	0.69	0.69	0.70

Figure 3. Relative abundances of Bacteria community at family level in the sludge used as inoculum, at the end of AD and UP1, UP2, UP3, UP4 phases. Communities with a relative abundance ≥ 1% (in at least one sample) are reported. Table shows Shannon-Weaver (H') and Pielou's evenness (E) diversity indices.

Anaerobaculaceae was the unique family within *Synergistetes* detected in all samples. It was dominant in the community of inoculum (31%) and it slightly decreased toward the AD (19%) and UP1 phase (23%). It reverted to the inoculum abundance at the end of UP2 and UP3 experimental phases (34% and 31%, respectively), while slightly decrease during UP4 phase (21%). This family was identified as *Anaerobaculum* at genus level but it was not affiliated at specie level. *Anaerobaculum* genus was already detected in our CSTR during a previous experimental period (Lembo et al., 2021) characterized by a 15 days of HRT, and it was identified as *A. hydrogeniformans* specie, a NaCl requiring fermentative bacterium suitable for the saline and the milk derived substrates. The same microorganism was identified by Treu et al. (2019) during thermophilic anaerobic digestion of cheese whey as well as by Fontana et al.(2018) in a thermophilic single-stage CSTR treating cheese wastes and working at 15 days of HRT (Fontana et al., 2018a; Treu et al., 2019).

Among *Firmicutes* phylum the highest number of families were identified. An unidentified family belonging to the recently discovered MBA08 order of the Clostridia class, was identified in the inoculum (11%). It after decreased to 5% during AD phase while it progressively increased during UP1 (7%), UP2 (8%), UP3 (14%) and UP4 (20%). The high abundance of this microorganism, which has anaerobic carbohydrate-fermenting characteristics(Kougias et al., 2017) is clearly aligned with other studies on biological biogas upgrading systems (Porté et al., 2019). However, the difficulty of assigning this group in lower taxonomic classification levels highlights its importance as novel microbe residing in engineered AD ecosystems during hydrogen assisted methanogenesis.

Members of the *Lachnospiraceae* family were present in the inoculum (4%), they increased significantly during AD phase (16%) and they after decreased in UP1 and UP2 (1% and 2%, respectively) until disappearing in UP3 and UP4. Increase of relative abundance in AD phase is in accordance with our previous results (Lembo et al., 2021).This family was characterized at specie level as *Defluviitale*a *saccarophila* , a thermophilic fermenting bacteria producing acetate, formate, n-butyrate and butyrate as the main products (Jabari et al., 2012).

Moreover, minor representatives of *Clostridiaceae*, *Peptococcaceae* *Ruminococcaceae*, *Natranaerobiales* and *Caldicoprobacteraceae* families all belonging to *Clostridia* class, were identified. They can be considered as a group of microorganisms involved in various function of the AD process ranging from the hydrolysis of substrates to syntrophic acetate oxidation (SAO) activity. This group was identified in the inoculum (5%), during AD phase (3%) and in UP1 (6%) and UP3 (3%), UP2 (6%), UP3 (4%) and UP4 (3%) phases.

Among *Bacteroidetes* phylum, members of an unidentified family belonging to *Bacteriodales* order were found in inoculum (9%), AD (17%), UP1 (12%), UP2 (7%), UP3 (7%) and UP4 (9%) samples. *Bacteroidetes* is one of the most abundant phylum in biogas reactor (usually 10%) frequently involved in the hydrolytic step of AD (Fontana et al., 2018a). Interestingly, this phylum represented only a small fraction of the microbial community identified during the AD phase in both the effluent and the interstitial matrix of GSTR during our previous experimentation (Lembo et al., 2021), and it completely disappeared during the hydrogen injection phases. In the current experimentation it remained stable into GSTR and it was identified in all samples. According to Campanaro et al. (Campanaro et al., 2016), *Bacteroidetes* were commonly founded in mesophilic and thermophilic reactors treating manure and their abundances were inversely correlated with high values of HRT. Therefore, it is possible that *Bacteroidetes* microorganisms were favoured in the GSTR by the HRT of 30 days, which corresponded to the double of the HRT previously used (Lembo et al., 2021). Moreover, *Porphyromonadaceae* family was only detected in inoculum sample (3%) and it disappeared during all experimental phases.

The *Thermotogaceae* family was the unique member of the *Thermotogae* phylum and it was characterized as *S1* at genus level in the inoculum (5%), during the AD phase (7%) and during the hydrogen injection in UP1 (11%), UP2 (13%), UP3 (5%) and UP4 (4%) samples. It has a role in the SAO activity in mutualistic cooperation with hydrogenotrophic methanogens (Li et al., 2018).

*Pirellulaceae (*order *Pirellulales)* was the unique family within *Planctomyces* which was detected in the sludge used as inoculum (9%) and it was quite stable throughout the AD, UP1, UP2, UP3 and UP4 experimental phases with a similar relative abundances (8%, 10%, 9%, 12% and 9%, respectively). *Planctomyces* community is scarcely described in literature and it is always represented as a minor group of mesophilic reactors (Nelson et al., 2011; Rivière et al., 2009). An unidentified family belonging to *Planctomyces* phylum was detected in the GSTR during previous experimental periods (Lembo et al., 2021) in both the effluent and the interstitial matrix after insertion of H_2 flux and biogas recirculation. Since it was detected in the sludge used as inoculum, we can hypothesize that in the current study members of the *Pirellulaceae* family were present also in the effluent of AD phase probably favored by both the longer HRT and the lower ORL used, as suggested by Krakat at al. (Krakat et al., 2011). A similar behavior is showed by the SHA-31 family (class *Anaerolineae*) as representative of *Chloroflexi* phylum. Indeed, members of SHA31 were not identified in the AD phase of our previous experimentation (Lembo et al., 2021) but they are present in the current study with a 4% of relative abundance which increased to 12%, 9%,7% and 14% in the UP1, UP2, UP3 and UP4 samples, respectively. Since a small community showing a

relative abundance <1% was also identified in the sludge used as inoculum, we can hypothesize that members of SHA-31 family were stabilised during AD process by the longer HRT used as compared to our previous activities. A semi-syntrophic activity in cooperation with hydrogenotrophic methanogens is attributed to members of SHA-31 family (Lembo et al., 2021). Finally, it is worth noting the presence of *Pseudomonadaceae* family as peculiar microbial group of the AD sample (7%). Members of this family belong to the *fragi* specie and are characterized by the role of accelerating the electron transfer among syntrophic bacteria and methanogens (Kougias et al., 2017). This microorganisms decreased significantly when the hydrogen was introduced *in situ* to the GSTR, suggesting at the same time an adaptation of the microbial pathway involved in methanogenesis.

According to both Shannon-Weaver (H') and Pielou's Evenness (E) indices, the diversity of Bacteria communities in the inoculum (H'=2.51 and E=0.75) and during AD (H'=2.37 and E=0.74), UP1 (H'=2.37 and E=0.75), UP2 (H'=2.20 and E=0.69) UP3 (H'=2.26 and E=0.69) and UP4 (H'=2.26 and E=0.70) phases, was very similar. This is not surprisingly, since the sludge used as inoculum was characterized by an already acclimated microbial community to the AD process with SCW as well as to the hydrogen injection during our previous activities (Lembo et al., 2021). Though this trend, it is worth noting that the higher diversity was observed between the sludge used as inoculum and the UP4 sample. In the latter, both indexes decreased suggesting the development of a more uniform distribution of a specialised community.

3.3.5 Archaea communities

Archaea microbial communities were represented by the unique phylum of *Euryarchaeota.* Relative abundances at family and genus level are shown in Figure 4.

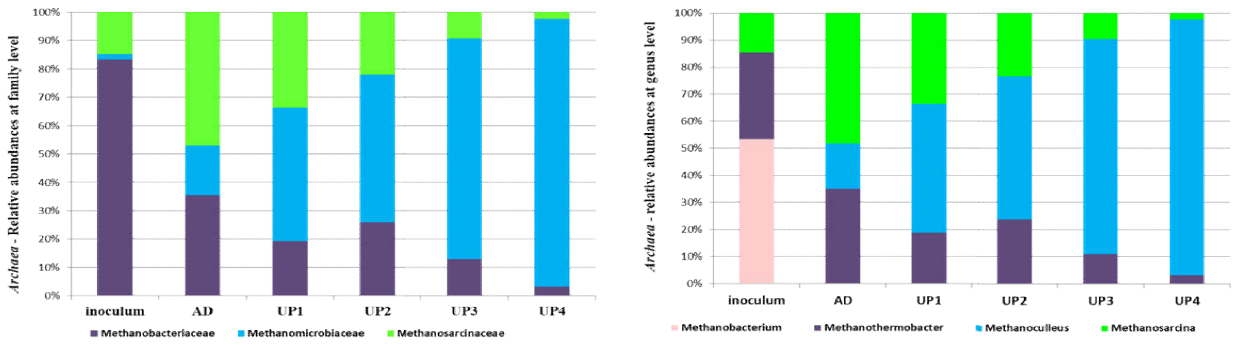


Figure 4. Relative abundance of the archaea community at the family and genus level in the sludge used as inoculum, at the end of AD and UP1, UP2, UP3, UP4 phases. Communities with a relative abundance >1% in at least one sample are reported

Members of *Methanobacteriaceae* (83%) dominated in the sludge used as inoculum and they corresponded to a core population of two methanogens : *Methanotermobacter* (32%) and *Methanobacterium* (53%) genera with the latter identified as *thermautotrophicus* at specie level. Therefore, a dominant hydrogenotrophic pathway characterised the sludge used as inoculum. According to Treu, this pathway is driven by the saline characteristic of cheese whey. Moreover, members of the *Methanosarcinaceae* family were identified at low abundance (15%) and they corresponded to *Methanosarcina* genus. *Methanosarcina* microorganisms are considered as stabilizer of AD process due to the ability of switching their pathway from acetoclastic to hydrogenotrophic methanogenesis as, for example, when H_2 partial pressure increases (Braga Nan et al., 2020). Finally, the inoculum was characterised by a small community of methanogens belonging to *Methanomicrobiaceae* (2%).

The archaea community significantly changed during AD phase: *Methanobacteriaceae* family dropped to 35% while both *Methanosarcinaceae* and *Methanomicrobiaceae* increased to 47% and 17%, respectively. *Methanotermobacter thermautotrophicus* was the unique specie identified within *Methanobacteriaceae,* while *Methanothermobacter* (unidentified at specie level) disappeared. All microorganisms of the *Methanosarcinaceae* and *Methanomicrobiaceae* families were grouped in the respective *Methanosarcina* and *Methanoculleus* genera and both methanogens were not identified at species level. The main changes observed in the community of methanogens during AD phase highlighted that a new hydrogenotrophic community (*Methanoculleus*) replaced the previous one (*Methanobacterium*) and that the acetoclastic *Methanosarcina* community took an advantage over this shift. Both structural and functional shifts in the archaea defined a community equally distributed between hydrogenotrophic and acetoclastic methanogens producing a very stable AD process. Interestingly, in our previous activities (Lembo et al., 2021) the methanogens community was entirely represented by the hydrogenotroph *M. thermautotrophicus*, while *Methanosarcina* and *Methanoculleus* methanogens were identified in the interstitial matrix of the immobilized phase with very low abundances. The methanogens community was further modified by the hydrogen introduction to the GSTR (UP1- UP4 phases). Methanogens of the *Methanoculleus* genus progressively increased in UP1 (47%) and UP2 (51%) until they became dominant in UP3 (77%) and UP4 (94%). At the same time, members of *Methanotermobacter* decreased to 19%, 23%, 10% and 3% in UP1, UP2, UP3 and UP4, respectively and members of *Methanosarcina decreased* to 33%, 22%, 9% and 2% UP1, UP2, UP3 and UP4, respectively. The dominance of the hydrogenotrophic *Methanoculleus* genus characterized the archaea community at the end of the upgrading experimental phase, in agreement with other studies that revealed its presence in the effluent of digesters (Kougias et al., 2017; Treu et al., 2019), but also in the methanogenic biofilm

grown in the carrier material inside digesters (Maegaard et al., 2019). *Methanoculleus* dominance in our experimental system was probably favoured by the higher amount of hydrogen that was supplied to the GSTR. Indeed, it is known that the increase of hydrogen partial pressure due to the injection of exogenous H_2 can modify the thermodynamic equilibrium promoting metabolic other pathways that act as sink of H_2 utilization (Kougias et al., 2020). Moreover, during UP3 and UP4 phases, the acetoclastic community of *Methanosarcina* methanogens significantly decreased and acetate accumulated in GSTR (Table 3).

3.4 Conclusion

The current research showed the potential of the GSTR configuration to perform *in situ* biomethanation by increasing gas recirculation rates. The higher MER value of 0.29 L_{CH4} L_r^{-1} d^{-1} was obtained at gas recirculation rate of 235 L L_r^{-1} d^{-1}. The organic matter removal was not compromised by H_2 supply but the increase of acetate concentration was observed.

The composition of the gas outflow showed a CH_4, H_2 and CO_2 content of 75%, 20.5% and 4.3%, respectively. Lower MER value (0.16 L_{CH4} L_r^{-1} d^{-1}) and comparable CH_4 content (73%) were obtained by a CSTR configuration implemented with a hollow fibre-membrane module submerged in the reactor and by gas recirculation. Then, the immobilization strategy used in this study can have played a significant role in extending gas-liquid contact area.

The Bacteria community characterising the sludge used as inoculum, was able to easily adapt to the GSTR, and it remained quite stable during AD, UP1 and UP4 phases. The most relevant changes characterised the methanogenic community. During AD phase, a mixed community of both acetoclastic and hydrogenotrophic methanogens evolved. Addition of exogenous hydrogen during the biomethanation phases produced a metabolic shift from a mixed community of acetoclastic/hydrogenotrophic methanogens toward the dominance of the hydrogenotrophic *Methanoculleus* genus. Therefore, in the current study the methanogenic community was boosted by the *in situ* biomethanation process.

Further research are needed to elucidate the exact role of the immobilized area in the gas-liquid mass transfer. Moreover, a deeper insight into both the role and the function of the microbial community inside the immobilized area will help us to understand the syntrophic relationships regulating the *in situ* biomethanation.

3.5 References

Agneessens, L.M., Ottosen, L.D.M., Andersen, M., Berg Olesen, C., Feilberg, A., Kofoed, M.V.W., 2018. Parameters affecting acetate concentrations during *in-situ* biological hydrogen methanation. Bioresour. Technol. 258, 33–40.

Agneessens, L.M., Ottosen, L.D.M., Voigt, N.V., Nielsen, J.L., de Jonge, N., Fischer, C.H., Kofoed, M.V.W., 2017. *In-situ* biogas upgrading with pulse H_2 additions: The relevance of methanogen adaption and inorganic carbon level. Bioresour. Technol. 233, 256–263.

Ahmad, T., Aadil, R.M., Ahmed, H., Rahman, U. ur, Soares, B.C.V., Souza, S.L.Q., Pimentel, T.C., Scudino, H., Guimarães, J.T., Esmerino, E.A., Freitas, M.Q., Almada, R.B., Vendramel, S.M.R., Silva, M.C., Cruz, A.G., 2019. Treatment and utilization of dairy industrial waste: A review. Trends Food Sci. Technol. 88, 361–372.

Alfaro, N., Fdz-Polanco, M., Fdz-Polanco, F., Díaz, I., 2019. H_2 addition through a submerged membrane for *in-situ* biogas upgrading in the anaerobic digestion of sewage sludge. Bioresour. Technol. 280, 1–8.

Ali Shah, F., Mahmood, Q., Maroof Shah, M., Pervez, A., Ahmad Asad, S., 2014. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. Sci. World J. 2.

Amann, R., Binder, B., Olson, R., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Envir. Microbiol. 56, 1919–1925.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-olignucleotide probing of whole cells for determinitive, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. Biotechnol. Adv. 36, 452–466.

Angelidaki, I., Xie, L., Luo, G., Zhang, Y., Oechsner, H., Lemmer, A., Munoz, R., Kougias, P.G., 2019. Biogas upgrading: Current and emerging technologies, Biomass, Biofuels, Biochemicals: Biofuels: Alternative Feedstocks and Conversion Processes for the Production of Liquid and Gaseous Biofuels.

Aryal, N., Kvist, T., Ammam, F., Pant, D., Ottosen, L.D.M., 2018. An overview of microbial biogas enrichment. Bioresour. Technol. 264, 359–369.

Asunis, F., Gioannis, G. De, Dessì, P., Isipato, M., Lens, P.N.L., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2020. The dairy biorefinery : Integrating treatment processes for cheese whey valorisation. J. Environ. Manage. 276, 111240.

Awe, O.W., Zhao, Y., Nzihou, A., Minh, D.P., Lyczko, N., 2017. A Review of Biogas Utilisation, Purification and Upgrading Technologies. Waste and Biomass Valorization 8, 267–283.

Baek, G., Kim, Jaai, Kim, Jinsu, Lee, C., 2018. Role and potential of direct interspecies electron transfer in anaerobic digestion. Energies 11, 107–125.

Barra Caracciolo, A., Grenni, P., Cupo, C., 2005. *In situ* analysis of native microbial communities in complex samples with high particulate loads. FEMS Microbiol. 253, 55–58.

Bassani, I., Kougias, P.G., Angelidaki, I., 2016. *In-situ* biogas upgrading in thermophilic granular UASB reactor: key factors affecting the hydrogen mass transfer rate. Bioresour. Technol. 221, 485–491.

Bassani, I., Kougias, P.G., Treu, L., Angelidaki, I., 2015. Biogas Upgrading via Hydrogenotrophic Methanogenesis in Two-Stage Continuous Stirred Tank Reactors at Mesophilic and Thermophilic Conditions. Environ. Sci. Technol. 49, 12585–12593.

Bassani, I., Kougias, P.G., Treu, L., Porté, H., Campanaro, S., Angelidaki, I., 2017. Optimization of hydrogen dispersion in thermophilic up-flow reactors for *ex situ* biogas upgrading. Bioresour. Technol. 234, 310–319.

Braga Nan, L., Trably, E., Santa-Catalina, G., Bernet, N., Delgenès, J.P., Escudié, R., 2020. Biomethanation processes: New insights on the effect of a high H_2 partial pressure on microbial communities. Biotechnol. Biofuels 13, 1–18.

British Petroleum Report, 2018. 67 th Statistical Review of World Energy.

Burkhardt, M., Busch, G., 2013. Methanation of hydrogen and carbon dioxide. Appl. Energy 111, 74–79.

Campanaro, S., Treu, L., Kougias, P.G., De Francisci, D., Valle, G., Angelidaki, I., 2016. Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnol. Biofuels 9, 26.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat. Publ. Gr. 7, 335–336.

Carvalho, F., Prazeres, A.R., Rivas, J., 2013. Cheese whey wastewater: Characterization and treatment. Sci. Total Environ. 445–446, 385–396.

De Francisci, D., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2015. Microbial diversity and dynamicity of biogas reactors due to radical changes of feedstock composition. Bioresour. Technol. 176, 56–64.

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Rev. Environ. Sci. Biotechnol. 7, 173–190.

Demirel, B., Yenigun, O., Onay, T.T., 2005. Anaerobic treatment of dairy wastewaters: A review. Process Biochem. 40, 2583–2595.

Diamantis, V.I., Kapagiannidis, A.G., Ntougias, S., Tataki, V., Melidis, P., Aivasidis, A., 2014. Two-stage CSTR-UASB digestion enables superior and alkali addition-free cheese whey treatment. Biochem. Eng. J. 84, 45–52.

Díaz, I., Pérez, C., Alfaro, N., Fdz-Polanco, F., 2015. A feasibility study on the bioconversion of CO_2 and H_2 to biomethane by gas sparging through polymeric membranes. Bioresour. Technol. 185, 246–253.

Dinamarca, C., Bakke, R., 2012. Simultaneous hydrogen production and consumption in Anaerobic mixed culture fermentation. Int. J. Energy Environ. 3, 323–332.

Dinamarca, C., Gañán, M., Liu, J., Bakke, R., 2011. H_2 consumption by anaerobic non-methanogenic mixed cultures. Water Sci. Technol. 63, 1582–1589.

European Biogas Association, 2018. EBA Statistical Report 2018. Annu. Rep. 68.

Faisal, S., Salama, E.S., Hassan, S.H.A., Jeon, B.H., Li, X., 2020. Biomethane enhancement via plastic carriers in anaerobic co-digestion of agricultural wastes. Biomass Convers. Biorefinery.

Felchner-Zwirello, M., Winter, J., Gallert, C., 2013. Interspecies distances between propionic acid degraders and methanogens in syntrophic consortia for optimal hydrogen transfer. Appl. Microbiol. Biotechnol. 97, 9193–9205.

Fernández, C., Cuetos, M.J., Martínez, E.J., Gómez, X., 2015. Thermophilic anaerobic digestion of cheese whey: Coupling H 2 and CH 4 production. Biomass and Bioenergy 81, 55–62.

Ferraro, A., Massini, G., Mazzurco Miritana, V., Rosa, S., Signorini, A., Fabbricino, M., 2020. A novel enrichment approach for anaerobic digestion of lignocellulosic biomass: Process performance enhancement through an inoculum habitat selection. Bioresour. Technol. 313, 123703–123712.

Ferry, J.G., 2010. The chemical biology of methanogenesis. Planet. Space Sci. 58, 1775–1783.

Fontana, A., Campanaro, S., Treu, L., Kougias, P.G., Cappa, F., Morelli, L., Angelidaki, I., 2018a. Performance and genome-centric metagenomics of thermophilic single and two-stage anaerobic digesters treating cheese wastes. Water Res. 134, 181–191.

Fontana, A., Kougias, P.G., Treu, L., Kovalovszki, A., Valle, G., Cappa, F., Morelli, L., Angelidaki, I., Campanaro, S., 2018b. Microbial activity response to hydrogen injection in thermophilic anaerobic digesters revealed by genome-centric metatranscriptomics. Microbiome 6, 1–14.

Gagliano, M.C., Braguglia, C.M., Petruccioli, M., Rossetti, S., 2015. Ecology and biotechnological potential of the thermophilic fermentative Coprothermobacter spp. FEMS Microbiol. Ecol. 91, 1–12.

Ghaib, K., Ben-Fares, F.Z., 2018. Power-to-Methane: A state-of-the-art review. Renew. Sustain. Energy Rev. 81, 433–446.

Ghosh, S., Chowdhury, R., Bhattacharya, P., 2016. Mixed consortia in bioprocesses: role of microbial interactions. Appl. Microbiol. Biotechnol. 100, 4283–4295.

Goli, A., Shamiri, A., Khosroyar, S., Talaiekhozani, A., Sanaye, R., Azizi, K., 2019. A review on different aerobic and anaerobic treatment methods in dairy industry wastewater. J. Environ. Treat. Tech. 7, 113–141.

Gorrasi, S., Izzo, G., Massini, G., Signorini, A., Barghini, P., Fenice, M., 2014. From polluting seafood wastes to energy. production of hydrogen and methane from raw chitin material by a two-phase process. J. Environ. Prot. Ecol. 15, 526–536.

Götz, M., Lefebvre, J., Mörs, F., McDaniel Koch, A., Graf, F., Bajohr, S., Reimert, R., Kolb, T., 2016. Renewable Power-to-Gas: A technological and economic review. Renew. Energy 85, 1371–1390.

Guebitz, G.M., Bauer, A., Bochmann, G., Gronauer, A., Weiss, S., 2015. Biogas Science and Technology, Biogas Science and Technology.

Gujer, W., Zehnder, A.J.B., 1983. Conversion processes in anaerobic digestion. Water Sci. Technol. 15, 127–167.

Hattori, S., 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. 23, 118–127.

Ho, D., Jensen, P., Batstone, D., 2014. Effects of temperature and hydraulic retention time on acetotrophic pathways and performance in high-rate sludge digestion. Environ. Sci. Technol. 48, 6468–6476.

Hulshoff Pol, L.W., De Castro Lopes, S.I., Lettinga, G., Lens, P.N.L., 2004. Anaerobic sludge granulation. Water Res. 38, 1376–1389.

Ishii, S., Kosaka, T., Hotta, Y., Watanabe, K., 2006. Simulating the contribution of coaggregation to interspecies hydrogen fluxes in syntrophic methanogenic consortia. Appl. Environ. Microbiol. 72, 5093–5096.

Izzo, G., Rosa, S., Massing, G., Patriarca, C., Fenice, M., Fiocchetti, F., Marone, A., Varrone, C., Signorini, A., 2014. From hypertrophic lagoons to bioenergy production. J. Environ. Prot. Ecol. 15, 537–546.

Jabari, L., Gannoun, H., Cayol, J.L., Hamdi, M., Fauque, G., Ollivier, B., Fardeau, M.L., 2012. Characterization of defluviitalea saccharophila gen. nov., sp. nov., a thermophilic bacterium isolated from an upflow anaerobic filter treating abattoir wastewaters, and proposal of defluviitaleaceae fam. nov. Int. J. Syst. Evol. Microbiol. 62, 550–555.

Kapoor, R., Ghosh, P., Kumar, M., Vijay, V.K., 2019. Evaluation of biogas upgrading technologies and future perspectives: a review. Environ. Sci. Pollut. Res. 26, 11631–11661.

Karadag, D., Köroılu, O.E., Ozkaya, B., Cakmakci, M., 2015. A review on anaerobic biofilm reactors for the treatment of dairy industry wastewater. Process Biochem. 50, 262–271.

Kato, S., Hashimoto, K., Watanabe, K., 2012. Microbial interspecies electron transfer via electric currents through conductive minerals. Proc. Natl. Acad. Sci. U. S. A. 109, 10042–10046.

Khemkhao, M., Nuntakumjorn, B., Techkarnjanaruk, S., Phalakornkule, C., 2011. Effect of chitosan on UASB treating POME during a transition from mesophilic to thermophilic conditions. Bioresour. Technol. 102, 4678–4681.

Koller, M., Gasser, I., Schmid, F., Berg, G., 2011. Linking ecology with economy: Insights into polyhydroxyalkanoate-producing microorganisms. Eng. Life Sci. 11, 222–237.

Kougias, P.G., Angelidaki, I., 2018. Biogas and its opportunities—A review. Front. Environ. Sci. Eng. 12, 1–12.

Kougias, P.G., Treu, L., Benavente, D.P., Boe, K., Campanaro, S., Angelidaki, I., 2017. *Ex-situ* biogas upgrading and enhancement in different reactor systems. Bioresour. Technol. 225, 429–437.

Krakat, N., Schmidt, S., Scherer, P., 2011. Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. Bioresour. Technol. 102, 5692–5701.

Lane, N., Martin, W.F., Raven, J.A., Allen, J.F., 2013. Energy, genes and evolution: Introduction to an evolutionary synthesis. Philos. Trans. R. Soc. B Biol. Sci. 368, 1–5.

Lecker, B., Illi, L., Lemmer, A., Oechsner, H., 2017. Biological hydrogen methanation – A review. Bioresour. Technol. 245, 1220–1228.

Lembo, G., Massini, G., Mazzurco Miritana, V., Fenice, M., Felici, C., Liberatore, R., Signorini, A., 2016. Anaerobic digestion of ricotta cheese whey : effect of phase separation on methane production and microbial community structure, in: European Biomass Conference and Exhibition Proceedings, 2016 (24thEUBCE). ETA-Florence Renewable Energies, pagg. 599–607.

Lembo, G., Rosa, S., Miritana, V.M., Marone, A., Massini, G., Fenice, M., Signorini, A., 2021. Thermophilic anaerobic digestion of second cheese whey: Microbial community response to H_2 addition in a partially immobilized anaerobic hybrid reactor. Processes 9, 1–16.

Li, D., Ran, Y., Chen, L., Cao, Q., Li, Z., Liu, X., 2018. Instability diagnosis and syntrophic acetate oxidation during thermophilic digestion of vegetable waste. Water Res. 139, 263–271.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171–189.

Luo, G., Angelidaki, I., 2013a. Hollow fiber membrane based H_2 diffusion for efficient *in situ* biogas upgrading in an anaerobic reactor. Appl. Microbiol. Biotechnol. 97, 3739–3744.

Luo, G., Angelidaki, I., 2013b. Co-digestion of manure and whey for *in situ* biogas upgrading by the addition of H_2: Process performance and microbial insights. Appl. Microbiol. Biotechnol. 97, 1373–1381.

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., Angelidaki, I., 2012. Simultaneous hydrogen utilization and *in situ* biogas upgrading in an anaerobic reactor. Biotechnol. Bioeng. 109, 1088–1094.

Maegaard, K., Garcia-Robledo, E., Kofoed, M.V.W., Agneessens, L.M., de Jonge, N., Nielsen, J.L., Ottosen, L.D.M., Nielsen, L.P., Revsbech, N.P., 2019. Biogas upgrading with hydrogenotrophic methanogenic biofilms. Bioresour. Technol. 287, 121422.

Mainardis, M., Flaibani, S., Trigatti, M., Goi, D., 2019. Techno-economic feasibility of anaerobic digestion of cheese whey in small Italian dairies and effect of ultrasound pre-treatment on methane yield. J. Environ. Manage. 246, 557–563.

Martin, M.R., Fornero, J.J., Stark, R., Mets, L., Angenent, L.T., 2013. A single-culture bioprocess of methanothermobacter thermautotrophicus to upgrade digester biogas by CO_2-to-CH_4 conversion with H_2. Archaea 2013, 157529.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J.M., Schink, B., Rohlin, L., Gunsalus, R.P., 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Ann. N. Y. Acad. Sci. 1125, 58–72.

Mulat, D.G., Mosbæk, F., Ward, A.J., Polag, D., Greule, M., Keppler, F., Nielsen, J.L., Feilberg, A., 2017. Exogenous addition of H_2 for an *in situ* biogas upgrading through biological reduction of carbon dioxide into methane. Waste Manag. 68, 146–156.

Muñoz, R., Meier, L., Diaz, I., Jeison, D., 2015. A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Rev. Environ. Sci. Biotechnol. 14, 727–759.

Nakasaki, K., Nguyen, K.K., Ballesteros, F.C., Maekawa, T., Koyama, M., 2020. Characterizing the microbial community involved in anaerobic digestion of lipid-rich wastewater to produce methane gas. Anaerobe 61, 102082.

Narayanan, C.M., Narayan, V., 2019. Biological wastewater treatment and bioreactor design: A review. Sustain. Environ. Res. 1, 1–17.

Narihiro, T., Terada, T., Ohashi, A., Kamagata, Y., Nakamura, K., Sekiguchi, Y., 2012. Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment systems by sequence-specific rRNA cleavage method. Water Res. 46, 2167–2175.

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. 102, 3730–3739.

O'Flaherty, V., Collins, G., Mahony, T., 2006. The microbiology and biochemistry of anaerobic bioreactors with relevance to domestic sewage treatment. Rev. Environ. Sci. Biotechnol. 5, 39–55.

Patterson, T., Esteves, S., Dinsdale, R., Guwy, A., Maddy, J., 2013. Life cycle assessment of biohydrogen and biomethane production and utilisation as a vehicle fuel. Bioresour. Technol. 131, 235–245.

Pavan, M.E., Pavan, E.E., Glaeser, S.P., Etchebehere, C., Kämpfer, P., Pettinari, M.J., López, N.I., 2018. Proposal for a new classification of a deep branching bacterial phylogenetic lineage: Transfer of Coprothermobacter proteolyticus and Coprothermobacter platensis to Coprothermobacteraceae fam. nov., within Coprothermobacterales ord. nov., Coprothermobacte. Int. J. Syst. Evol. Microbiol. 68, 1627–1632.

Pervin, H.M., Dennis, P.G., Lim, H.J., Tyson, G.W., Batstone, D.J., Bond, P.L., 2013. Drivers of microbial community composition in mesophilic and thermophilic temperature-phased anaerobic digestion pre-treatment reactors. Water Res. 47, 7098–7108.

Pohland, F.G., Ghosh, S., 1971. Developments in anaerobic stabilization of organic wastes - the two-phase concept. Environ. Lett. 1, 255–266.

Porté, H., Kougias, P.G., Alfaro, N., Treu, L., Campanaro, S., Angelidaki, I., 2019. Process performance and microbial community structure in thermophilic trickling biofilter reactors for biogas upgrading. Sci. Total Environ. 655, 529–538.

R.I. Amann, K.H. Ludwig, K.H.S., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Mol. Biol. Rev. 24, 143–169.

Rachbauer, L., Voitl, G., Bochmann, G., Fuchs, W., 2016. Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. Appl. Energy 180, 483–490.

Rajendran, K., Mahapatra, D., Venkatraman, A.V., Muthuswamy, S., Pugazhendhi, A., 2020. Advancing anaerobic digestion through two-stage processes: Current developments and future trends. Renew. Sustain. Energy Rev. 123, 109746.

Ramasamy, E.., Abbassi, S.., 2000. Energy recovery from dairy waste-waters: impacts of biofilm support systems on anaerobic CST reactors. Appl. Energy 65, 91–98.

Ramsay, I.R., Pullammanappallil, P.C., 2001. Protein degradation during anaerobic wastewater treatment: Derivation of stoichiometry. Biodegradation 12, 247–257.

Regueira, A., González-Cabaleiro, R., Ofiţeru, I.D., Rodríguez, J., Lema, J.M., 2018. Electron bifurcation mechanism and homoacetogenesis explain products yields in mixed culture anaerobic fermentations. Water Res. 141, 349–356.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3, 700–714.

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy 38, 13172–13191.

Sarker, S., Lamb, J.J., Hjelme, D.R., Lien, K.M., 2018. Overview of recent progress towards *in-situ* biogas upgradation techniques. Fuel 226, 686–697.

Schink, B., 2006. Syntrophic associations in methanogenic degradation. Prog. Mol. Subcell. Biol. 41, 1–19.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61, 262–280.

Shen, L., Zhao, Q., Wu, X., Li, X., Li, Q., Wang, Y., 2016. Interspecies electron transfer in syntrophic methanogenic consortia: From cultures to bioreactors. Renew. Sustain. Energy Rev. 54, 1358–1367.

Stams, A.J.M., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7, 568–577.

Sun, Q., Li, H., Yan, J., Liu, L., Yu, Z., Yu, X., 2015. Selection of appropriate biogas upgrading technology-a review of biogas cleaning, upgrading and utilisation. Renew. Sustain. Energy Rev. 51, 521–532.

Tandishabo, K., Nakamura, K., Umetsu, K., Takamizawa, K., 2012. Distribution and role of Coprothermobacter spp. in anaerobic digesters. J. Biosci. Bioeng. 114, 518–520.

Treu, L., Tsapekos, P., Peprah, M., Campanaro, S., Giacomini, A., Corich, V., Kougias, P.G., Angelidaki, I., 2019. Microbial profiling during anaerobic digestion of cheese whey in reactors operated at different conditions. Bioresour. Technol. 275, 375–385.

Turner, J., Sverdrup, G., Mann, M.K., Maness, P.C., Kroposki, B., Ghirardi, M., Evans, R.J., Blake, D., 2008. Renewable hydrogen production. Int. J. Energy Res. 32, 379–407.

Vasmara, C., Marchetti, R., 2017. Initial pH influences in-batch hydrogen production from scotta permeate. Int. J. Hydrogen Energy 42, 14400–14408.

Wang, W., Xie, L., Luo, G., Zhou, Q., Angelidaki, I., 2013. Performance and microbial community analysis of the anaerobic reactor with coke oven gas biomethanation and *in situ* biogas upgrading. Bioresour. Technol. 146, 234–239.

Xia, A., Cheng, J., Murphy, J.D., 2016. Innovation in biological production and upgrading of methane and hydrogen for use as gaseous transport biofuel. Biotechnol. Adv. 34, 451–472.

Xia, Y., Wang, Y., Fang, H.H.P., Jin, T., Zhong, H., Zhang, T., 2014. Thermophilic microbial cellulose decomposition and methanogenesis pathways recharacterized by metatranscriptomic and metagenomic analysis. Sci. Rep. 4, 1–9.

Yang, K., Yu, Y., Hwang, S., 2003. Selective optimization in thermophilic acidogenesis of cheese-whey wastewater to acetic and butyric acids : partial acidification and methanation 37, 2467–2477.

Yue, Z., Chen, R., Yang, F., MacLellan, J., Marsh, T., Liu, Y., Liao, W., 2013. Effects of dairy manure and corn stover co-digestion on anaerobic microbes and corresponding digestion performance. Bioresour. Technol. 128, 65–71.

Zabranska, J., Pokorna, D., 2018. Bioconversion of carbon dioxide to methane using hydrogen and hydrogenotrophic methanogens. Biotechnol. Adv. 36, 707–720.

Zhang, L., Kuroki, A., Tong, Y.W., 2020. A Mini-Review on *In situ* Biogas Upgrading Technologies via Enhanced Hydrogenotrophic Methanogenesis to Improve the Quality of Biogas From Anaerobic Digesters. Front. Energy Res. 8, 1–7.

Zhu, X., Chen, L., Chen, Y., Cao, Q., Liu, X., Li, D., 2019. Differences of methanogenesis between mesophilic and thermophilic *in situ* biogas-upgrading systems by hydrogen addition. J. Ind. Microbiol. Biotechnol. 46, 1569–1581.

Zotta, T., Solieri, L., Iacumin, L., Picozzi, C., Gullo, M., 2020. Valorization of cheese whey using microbial fermentations. Appl. Microbiol. Biotechnol. 104, 2749–2764.

4 Main conclusion of the thesis

The general aim of this thesis was the development and the study of the *in situ* biological biogas upgrading process by the selective enrichment of the hydrogenotrophic methanogenic community

The study evolved through several experimental phases, each with the aim of developing the process towards a wider application.

During the research period an innovative biological upgrading set- up, called Gas-Stirred Tank Reactor (GSTR), was designed and assessed. The proposed configuration is the result of the CSTR modification, in which about one third of the reactor's working volume (50L) was filled with polymeric supports (high-density polyethylene, HDPE) and a gas recirculation was installed for homogenization of nutrients and as a strategy to improve H_2 solubility. The thermophilic condition was selected to exploit the acclimatization of the mixed microbial community used as inoculum and collected from a thermophilic (55 °C) anaerobic digestion plant.

In addition, in order to avoid the increase of pH above the suitable range for DA process, it was decided to use as feed an easily fermentable substrate such as cheese whey, a by-product of the dairy industry, very abundant in Italy and Europe.

The principal aim was to evaluate the impacts of hydrogen addition on the microbial community structure involved in the process of anaerobic digestion (article 1), and then to evaluate the performance of the process in the GSTR system with respect to the conversion efficiency and the final methane content in the biogas (article 2).

Major results reported in article 1 are listed below:

- Data on microbial abundance (N.cell ml^{-1}) showed an increase in bacteria communities at in the interstitial matrix respect the effluent before and after H_2 addition
- the H_2 addition increased the *Archaea* communities in both effluent and interstitial matrix as compared with the phase without upgrading
- From a functional point of view, we identified a common microbial "core" in all samples represented by members of the *Anaerobaculaceae* family, involved to the SCW metabolism
- In addition, in samples collected from the interstitial matrix at the end of the experimental phases, a new microbial core was identified in members of the *Thermodesulfobiaceae*

family. Previous studies highlighted how these families carried out an acidogenic hydrolytic activity. Moreover, its involvement in the syntrophic acetate oxidization (SAO) pathway during thermophilic AD processes has been previously suggested

- H_2 addition caused the enrichment of a peculiar microbial core represented by members of the SHA-31 family of the class of *Anaerolineae.* This family is involved in heterotrophic carbohydrate degradation and in interspecies electron transfer mechanism in mutualistic cooperation with methanogens.
- A common core of the methanogenic microbial community was identified in the hydrogenotrophic members of the *Methanobacteriaceae* family in both phases, with and without H_2 addition.
- The versatile members of *Methanosarcinaceae* were enriched by the H_2 addition.
- These functional microbial cores allowed to obtain a balanced AD process in the different experimental periods as shown by results of the GSTR performances at the steady state. Therefore, the whole microbial communities were able to adapt to H_2, as well as to the HDPE supports, thus, maintaining all the metabolic syntrophic relationships over the experimental time.
- H_2 injection increased the CH_4 production rate by 25% as compared to what obtained during conventional AD, without compromising the efficiency of organic matter removal.
- The use of undiluted RCW as the only substrate ensured a stable anaerobic digestion process without the use of pH control.

Major results reported in article 2 are listed below:

- The GSTR reactor was suitable to optimize the *in situ* biomethanation process: a biogas with 75% of methane content was reached by increasing gas recirculation rates.
- Addition of exogenous hydrogen during the biomethanation phases produced a metabolic shift from a mixed community of acetoclastic/hydrogenotrophic methanogens toward the dominance of the hydrogenotrophic *Methanoculleus* genus.
- Moreover, the *Bacteria* community characterising the sludge used as inoculum, was able to easily adapt to the GSTR configuration, and it remained quite stable during the experimental phases.
- Members of *Anaerobaculaceae* family were attested as the RCW-specific microbial core.
- Members of SHA-31 family (Class *Anaerolineae*), were attested as the hydrogen-specific microbial core.

This PhD study also highlights the importance of considering the composition of outflow gas. Indeed, the high value of hydrogen in the gas mixture (20%), which suggested a H_2 mass gas-liquid transfer limitation, combined to the low CO_2 value (≈ 4%), would open up the possibility of using the proposed GSTR configuration to produce the hythane, a gaseous blend composed of 10%–30% v/v H_2 and 70%–90% v/v CH_4, and mainly used in the automotive sector in place of methane.

Further research are needed to elucidate the exact role of the immobilized area in the gas-liquid mass transfer. Moreover, a deeper insight into both the role and the function of the microbial community inside the immobilized area will help us to understand the syntrophic relationships regulating the *in situ* biomethanation.