

# Bio-production of Hydrogen and Methane Through Anaerobic Digestion Stages

Chiara Patriarca, Elena De Luca, Claudio Felici, Luigia Lona,  
Valentina Mazzurco Miritana and Giulia Massini

**Abstract** The anaerobic digestion (AD) is a widespread technology for the energetic valorization of organic wastes and the plants number is increasing both in industrialized and emerging countries. On the other hand, a low efficiency of the biogas production is still observed mainly due to the limited knowledge on the microorganism interactions and the lack of microbiological monitoring in the AD process. The energetic valorization of organic waste, producing both methane and hydrogen, can be increased by applying microbial ecology principles and exploiting the potential offered by functional microbial biodiversity.

**Keywords** Anaerobic digestion · Organic wastes · Microbial ecology

## List of Symbols

ADP	Adenosine DiPhosphate
ATP	Adenosine TriPhosphate
AD	Anaerobic Digestion
BChl	BacterioChlorophyll
GSB	Green Sulphur Bacteria
LED	Light-Emitting Diodes
NADH	Nicotinamide Adenine Dinucleotide
VFA	Volatile Fatty Acids

---

C. Patriarca (✉) · E. De Luca · C. Felici · L. Lona · V.M. Miritana · G. Massini  
ENEA, Italian National Agency for New Technologies, Energies and Sustainable  
Economic Development, Via Anguillarese, 301, 00123 Rome, Italy  
e-mail: chiara.patriarca@enea.it

L. Lona · V.M. Miritana  
Department of Ecology and Economic Development, Università della Tuscia, Largo  
dell'Università, 01100 Viterbo, Italy

## 1 Introduction

The consumption of energy resources, with a flow rate higher than their regeneration capacity, and the incomplete cyclization of industrial processes, has meant that within a few decades huge amounts of by-products and wastes arising from human activities have been produced: combustion gases, liquid pollutants, artefacts and solid compounds. The excess of waste materials and their irregular distribution in the geological compartments change the flow rate and the turnover by which the fundamental elements circulate, altering biogeochemical cycles and causing the phenomenon of pollution. It should also be added that the energy used by human activities is, in the end, lost to the environment as heat at a low temperature (entropy), contributing to the rapid change of the natural balances.

To counteract the current energy and environmental crisis as well as the acceleration of climate change caused by the use of fossil fuels, the scientific community is addressing the research on the diversification of the energy sources, focusing on sustainable alternative solutions, in particular bioenergy.

Within bioenergy, the production of biogas by anaerobic digestion (AD) of organic waste has emerged as one of the most promising process. It allows to produce bioenergy in form of methane, fulfilling the double goal of producing energy and valorizing organic wastes (agrozootechnical, agroindustrial and municipal organic materials) reducing its disposal costs. Moreover, the technology of the AD process is enough, simple and not very expensive.

In such a scenario it has also opened a lively debate on biohydrogen production. Hydrogen ( $H_2$ ), in fact, is produced and consumed during AD process, but new reactor configuration can allow to separate it before its consumption. Then it can be added to the biogas in order to increase its energetic value or it can be used alone.  $H_2$ , in fact, is considered the fuel of the future as its combustion produces more than three times the heat developed from fossil oil, while in terms of environmental sustainability the combustion process leads to the formation of only water vapour [13]. Therefore increasing the efficiency of  $H_2$  production within the dark fermentation stage may enhance methane production and the overall AD process. Recent research has been addressed towards a double-stage AD process consisting of a first fermentation stage for the  $H_2$  production and a second stage, feed with the effluent of the first one for the methane production. The use of a two-stage system should enhance the efficiency of the process, due to the fact that in the first reactor the fermentation allows the rapid production of organic acids and alcohols, which when released into the second stage would increase the production of methane.

## 2 Anaerobic Digestion Process

The AD process consists of a sequence of biochemical reactions carried out by various microorganisms that, in the absence of oxygen, transform organic substrates into simpler molecules, mainly acetic acid,  $H_2$ , methane ( $CH_4$ ) and carbon dioxide

(CO<sub>2</sub>). The content of CH<sub>4</sub> in the biogas varies depending on the characteristics of the organic matter, as well as by the process conditions and the reactor configuration. Although the methanogenic bacteria perform only the last step of the whole process, AD works as *an unicum* because the different groups of microorganism have evolved very close relationships of cooperation and the members of the food chain always depend on the earlier ones for their substrates.

Within AD, primary fermentation with H<sub>2</sub> production is due to an incomplete digestion process stopped before the methanogenic phase. The methanogenesis, in fact, consumes the amount of H<sub>2</sub> produced in the previous stages [45].

The techniques of AD can be divided into two main groups:

- Dry digestion when the substrate has a dry matter content higher than 20 %;
- Wet digestion when the substrate has a dry matter content lesser than 10 %, this is the most common technique, especially with the slurry.

The production of H<sub>2</sub> by dark fermentation is a natural biological process characteristic of the first two phases of AD (hydrolysis-acidogenesis) where bacteria hydrolyze the macromolecules involved and partially oxidize the organic substrates with the production of H<sub>2</sub> and CO<sub>2</sub>. The next two stages of AD (acetogenesis–methanogenesis) lead to the production of biogas, a mixture of 50–70 % CH<sub>4</sub> and 50–30 % for CO<sub>2</sub>.

## 2.1 *Hydrolysis*

The hydrolysis is the first phase of AD, during which complex organic molecules like polysaccharides, proteins, nucleic acids and lipids are split in their respective constituent oligomers and monomers. This phase is one of the most important processes: it may constitute a “bottleneck” for the entire process mainly when lignocellulosic substrates are used. In fact, not all the fermenting bacteria have high hydrolytic capacity and, on the other hand, the amount of substrate hydrolyzed contributes to determining the efficiency of the process.

## 2.2 *Acidogenesis*

It is considered the second phase of AD, during which the monomers that constitute the macromolecules are reduced into low molecular weight acids, the volatile fatty acids (VFA). Hydrolysis and acidogenesis perform the primary fermentation.

### 2.3 *The Fermentation*

In the absence of electron acceptors supplied from the environment, many organisms perform oxidation-reduction reactions of organic compounds internally balanced, with release of energy: this process is called fermentation.

The fermentation is an anaerobic metabolic process in which the donor and acceptor of electrons ( $e^-$ ) are organic molecules. In the course of the fermentation, an organic compound acts as a donor and oxidizes, while generating nicotinamide adenine dinucleotide (NADH) (i.e.  $\text{NAD}^+$  oxidized form). The NADH cannot unload its electrons in the transport chain; therefore to regenerate the pool of  $\text{NAD}^+$  necessary for the continuation of the process, the reoxidation of NADH occurs at the expense of an intermediate compound of the process, which acts as acceptor and the fermentation process is therefore a redox balanced in its interior.

When an organism degrades organic compounds have to face two main energetic metabolic issues:

- Eliminate the electrons removed from the electron donor;
- Store into adenosine triphosphate (ATP) part of the energy released during the process.

During the fermentation there is not the complete demolition of the starting compound, which is only partially fermented in one or more end products still mostly organic and which still retain part of the energy of the initial compound. Therefore, the energy yield of the fermentation is not comparable to that of respiratory processes and ATP, a phosphate compound of high energy, is generated only for the transfer of a phosphate group bonded to an intermediate of the process with a bond to adenosine diphosphate (ADP) (substrate-level phosphorylation).

During the fermentation the redox balance is maintained producing molecular  $\text{H}_2$ . Generally the production of  $\text{H}_2$  is associated with the presence in the organism of a protein called iron-sulphur ferredoxin, an electron carrier at low redox potential. The transfer of electrons from ferredoxin to  $\text{H}^+$  is catalyzed by the enzyme  $\text{H}_2$ ase. The diversity of fermentation and the metabolic pathways used by the bacteria lead to the formation of an alternative set of metabolic products and since  $\text{H}_2$  has the task of maintaining the redox balance, if the final products are more reduced, they result in a lower production of  $\text{H}_2$ . For example, when the ethanol, more reduced than acetate, is produced then  $\text{H}_2$  production will be less. Several bacteria produce acetate among the fermentation products. The production of acetate is energetically advantageous, because it allows the organism to produce additional ATP, always by substrate-level phosphorylation. The intermediate generated during the production of acetate is the acetyl-CoA, an intermediate high energy, which can be converted into acetylphosphate, where the phosphate group is transferred to ADP with the formation of ATP and acetate.

## 2.4 *Acetogenesis*

The third stage of the AD is represented by acetogenesis. In this phase, the volatile fatty acids previously produced are converted into acetic acid,  $\text{CO}_2$  and  $\text{H}_2$ . Acetogenesis is an interesting step, as it is performed by microorganisms that have evolved a syntrophic relationship with methanogenic bacteria so that that neither partner can operate without the other.

## 2.5 *Methanogenesis*

The production of  $\text{CH}_4$  is the conclusion of the AD process. Its production can take place essentially through two different pathways of reactions: a path provides  $\text{CH}_4$  by hydrogenotrophic bacteria that operate under the anaerobic oxidation of  $\text{H}_2$ , while the second pathway, the so-called acetoclastic, provides the dismutation of anaerobic acetic acid with formation of methane and carbon monoxide. In mesophylic condition, most of the  $\text{CH}_4$  production occurs through this second pathway. The quantity and composition of the biogas (at least in terms of  $\text{CH}_4$  and  $\text{CO}_2$ ) are of fundamental importance for the stability control of the AD process. If the reactor is operating under stable conditions, the production and composition of the biogas are constants. A decrease in the overall production of biogas and an increase in  $\text{CO}_2$  percentage may indicate phenomenal inhibition of the process. It follows that the production analysis and percentage composition of the biogas should always be associated with the control of parameters such as the concentration of volatile fatty acids.

## 3 **Dark Fermentation and Its Limiting Factors**

The production of  $\text{H}_2$  by dark fermentation is one of the oldest life processes; it is produced by many different microorganisms that are able to derive energy from organic substrates (heterotrophic). This means that heterotrophic organisms can also produce  $\text{H}_2$  from renewable sources, such as waste biomass from agricultural industry, the organic fraction of municipal waste, the wastewater from industrial and agricultural activities, offering the possibility of obtaining energy in an economically and ecologically even more sustainable way. In addition to reducing the cost of waste disposal, turning them into a resource, agricultural and food production processes or industrial processes are directed to closed circuits, which maximize the recycling of materials. It is a process that can take place at room temperature and pressure, without external energy input addition, so it is a process at positive energy balance.

Depending on the bacterial species, the organic substrates are employed and therefore the conditions of fermentation, the process leads to the formation of an

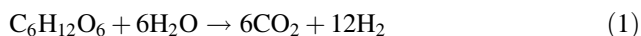
abundance of small organic molecules, VFA (butyrate, acetate, lactate, malate) and alcohols (ethanol, propanediol, butanediol) [44]. The theoretical yield (moles of H<sub>2</sub> produced per mole of substrate consumed) varies in function of the metabolic pathway, namely the organic acid/alcohol predominantly accumulated in the course of the fermentation, and is in turn the expression of the adaptations of the organisms themselves to “ecological factors” of the fermentation [20, 36].

The accumulation of these metabolites, on the one hand, constitutes a limiting factor for the production of H<sub>2</sub> but, on the other hand, they may confer an added value to the whole process as they have still a lot of energy in the form of chemical bonds. Depending on their nature, these molecules can be used as a substrate for further production of energy. Addressing this second step towards the H<sub>2</sub> production, the alternative possible solutions are:

- (a) photo-bioreactors in which purple non-sulphur bacteria produce H<sub>2</sub> in ambient light near infrared;
- (b) adoption of recent organic hydrolysis technology in which an electrolytic cell, with constructive features advanced (with bacteria adherent to the anode and cathode), stimulated with a potential difference of about 0.4 V, develops H<sub>2</sub>.

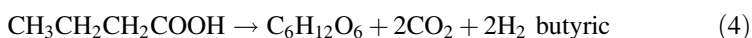
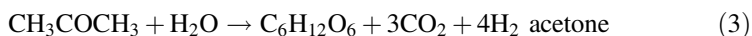
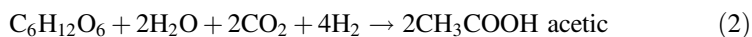
A more feasible alternative is given by the transfer of metabolites in reactors for the production of bio-methane, which is indicated by the EU as renewable energy sources and environmentally friendly energy that contributes to autonomy. This technology, which is consolidated and is now known on a large scale, was initially used with the objective of reducing the environmental impacts on air and soil originating from animal manure [58].

The maximum theoretical yield per mole of hexose by a complete conversion of glucose is 12 mol of H<sub>2</sub> [14]:

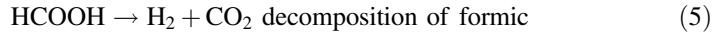


This would not, however, produce energy necessary for the growth of bacteria [19]. The yield of the process of production from glucose (moles of H<sub>2</sub>/moles substrate) varies depending of the final products that are formed, in fact, as previously mentioned, the H<sub>2</sub> is produced exclusively to balance the redox reaction, when the substrate is more or less reduced the formation of H<sub>2</sub> is less or more, respectively.

In the fermentation process acetic and butyric acid together with the production of acetone ensures the greatest accumulation of NADH corresponding to the increased productivity of the H<sub>2</sub>.



H<sub>2</sub> is also produced by the decomposition of formic acid, a by-product of acetic acid and alcoholic fermentation. Therefore the acetic acid fermentation appears to be the most promising for the synthesis of H<sub>2</sub> and ensures a potential productivity of 4 mol of H<sub>2</sub> per mole of glucose, while the butyric reaches a maximum of 2 mol.



### 3.1 *Main Process Parameters and Inhibition Factors*

In a mixed natural bacterial community the microorganisms have different enzyme complexes and may follow more than one metabolic pathway. Different parameters discriminate which becomes prevalent:

- pH
- Temperature
- H<sub>2</sub> partial pressure
- HRT (hydraulic retention time)
- VFA (volatile organic acids)
- resource mapping (quantity and type of fermentation substrate)
- concentration of metal ions.

### 3.2 *pH*

The fermentation medium acidity influences not only the biomass vitality conditions but also the type of products. The variation of the pH results in a variation of the electrostatic conditions (H<sup>+</sup> ions) which leads to a change in the conformation of the enzyme and the enzymatic activity responsible for the metabolic processes by reducing the catalytic action. It can lead to the transformation of nutrient substrates turning them into toxic substances.

Between pH 6–5.5 the proliferation of methanogenic bacteria (responsible for the consumption of H<sub>2</sub>) is prevented, but below pH 5 the productivity of H<sub>2</sub> is reduced causing a metabolic shift in favour of lactic acid. Furthermore, pH less than 4.6 converts the butyric fermentation in ethanolic reducing production yields [36].

Some studies have shown that a low initial pH (4–4.5) can cause a longer stationary phase compared to an initial high pH value [5]. However, the H<sub>2</sub> yield is lower with a high initial pH: in fact it causes a rapid production of H<sub>2</sub> and VFA that inhibit the buffering capacity and the bacteria cannot adapt to sudden environmental change [28].

### 3.3 Temperature

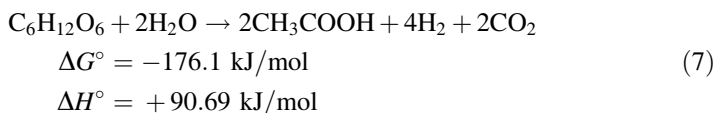
Temperature affects all physiological activities of microorganisms and the conversion rates of the fermentation products. The H<sub>2</sub> producing bacteria are very sensitive to temperature changing and the production rate undergoes various fluctuations before stabilizing at a given temperature [36].

All fermentation reactions can take place at mesophilic temperatures (25–40 °C), thermophilic (40–65 °C) or hyperthermophilic (>80 °C). In many studies the H<sub>2</sub> production is supported by mesophilic conditions and also in some thermophilic.

The effect of temperature on H<sub>2</sub> production can be explained thermodynamically by Van't Hoff equation

$$\ln K1/K2 = -\Delta H^\circ / R(1/T1 - T2) \quad (6)$$

Considering the Gibbs free energy and enthalpy of the glucose conversion to acetate



With increasing temperature the equilibrium constant kinetic increases, because the reaction is endothermic and thereby increases the concentration of H<sub>2</sub>.

In other studies it has been reported that the volumetric rate of H<sub>2</sub> production in thermophilic conditions was 60 % more compared to mesophilic conditions. Already at 37 °C, for example, it not only inhibits the activity of H<sub>2</sub> consumers, but also suppresses the growth of lactic acid bacteria [50].

However, high temperatures can cause thermic denaturation of proteins by damaging microbial activity and also with respect to the ambient temperature is higher than the energy cost.

### 3.4 Hydrogen Partial Pressure

Theoretical studies show that an increase of the H<sub>2</sub> partial pressure inhibits the same yield of H<sub>2</sub> production [20]. The H<sub>2</sub> partial pressure significantly influences its own production, especially when considering the continuous production, because when its concentration increases, the synthesis decreases. This is due to a mechanism of negative feedback inhibition, which produces other alternative metabolic pathways, leading to the synthesis of smaller substrates, such as lactate, ethanol, butanol or alanine. An increase of about 50 % of the production of H<sub>2</sub> was obtained in fact by removing the product from the headspace of the bioreactor through “sparge” nitrogen [32].



The  $H_2$  partial pressure has influence in the conversion rate of ethanol. It is well-known that ethanol can be accumulated only if the  $H_2$  partial pressure is greater than 104 Pa. However, when the  $H_2$  partial pressure is less than 104 Pa ethanol is converted into acetic acid.

The hydraulic retention time (HRT) retention time of the  $H_2$  phase is relatively short (24–72 h) with respect to that of the classic methanogenic reactors (15–40 d). This allows a significant reduction of the fermenter size among the methane one.

Some studies have shown that the degree of acidification during the fermentation process increases together with the HRT going from 28.2 to 59.1 % from 4 to 24 h. Moreover, the prolongation of HRT favours the biodegradability of some compounds; following the order of degradation carbohydrates > proteins > lipids [18].

The HRT also has significant effects on the metabolites distribution produced during the fermentation process as reported by Elefsiniotis and Oldham [17] and Henry et al. [21]. Indeed shorter retention times favour the production of propionate, but in general the total production of VFA/alcohol doubling from a hydraulic retention time of 4–12 h.

### 3.5 *Volatile Fatty Acids*

Volatile Fatty Acids are closely related to the pH of the system, able to select the fermentation process and also the production of  $H_2$  connected to the production of acids (particularly acetate and butyrate) by precise stoichiometric ratios. The same  $H_2$  formation matches with the formation of organic acids, the accumulation of which not only influences the rate of  $H_2$  production but may also change the fermentation pathways [41].

The anaerobic fermentation leads to the production of VFA and also to the formation of alcohols and reduced end products (involving the oxidation of NADH), such as ethanol, butanol and lactate containing  $H_2$  atoms withheld and not contributing to the  $H_2$  yield.

### 3.6 *Resource Mapping*

The type of substrate promotes the interspecific competition between the  $H_2$ -producing microorganisms, while the amount of substrate mainly determines intraspecific competition and helps to modify the enzymatic activity of bacteria causing a metabolic shift towards the production of lactic acid with a decrease of production yields.

The number of dominant populations at steady state depends on the number of types of substrates, however, this does not mean that the anaerobic fermentation of a particular substrate belong to just one type of fermentation. The metabolic

pathways may shift during fermentation with the self-metabolic activity of microorganisms that are adapted to the changes “ecosystem” [36].

### 3.7 Concentration of Metal Ions

Metal ions (Na, Mg, Zn, Fe) are essential micronutrients for bacterial metabolism, as they are required in the transport processes and enzymatic metabolism. For example, the Fe is involved in pyruvate oxidation to acetyl-CoA and in the proper action of the enzyme hydrogenase. Suboptimal concentrations of Fe ion can lead to shifts in metabolic pathways. When the concentration of Fe ions is very low in the fermentation medium of the enzymatic activity favours the production of lactic acid to ethanol by reducing the production of H<sub>2</sub>. Recent studies in the literature have shown that iron and nickel are elements essential for the production of gas by anaerobic microorganisms, since they are indispensable constituents of hydrogenase enzyme, which catalyzes the oxidation reaction of H<sub>2</sub> and reduction of protons during the fermentation process of degradation [6]. The results of different studies [34, 37, 64, 65, 68] have confirmed that the optimal dosage of nickel and iron to interior of the anaerobic system, as well as that inhibiting the production of H<sub>2</sub>, are closely dependent on the substrate, after inoculation and the operating conditions used in the digestion process.

## 4 Microbial Ecology and Syntrophic Cooperation Between Microorganism

Methanogenesis is a widespread process occurring mainly where primordial environment of Earth is retained. All the conditions are characterized by rich organic matter content and limited supply, not only of oxygen, but also of nitrate, sulphate, or oxidized iron or manganese species [38]. So when the electron acceptors with a decreasing energy yield are depleted [48], the degradation process becomes predominant. It is the least exergonic process in the degradation of organic matter and it is realized by complex networks of microorganisms that, operating subsequent reactions of oxidation and reduction, convert complex matter to its most oxidized state CO<sub>2</sub>, and its most reduced states CH<sub>4</sub> [47].

Compared with aerobic degradation or the alternative anaerobic respirations, methanogenic degradation is very poor in energy release; Shink stated that it gets back only 15 % of the energy that would be available in aerobic degradation, methane still having a high content of chemical energy.

From a metabolic point of view all methanogenic *Archaea* can be considered physiologically specialized as they produce methane using only a limited range of substrates, however simple, such as H<sub>2</sub>/CO<sub>2</sub>, formate and some methyl acetate compounds [38] relying on other organisms, belonging to the *Eubacteria* domain,

for the supply of this simple substrates. So the sequences of reactions that make possible the conversion of biomasses highly refractory to degradation like cellulose into  $\text{CH}_4$  and  $\text{CO}_2$ , is operated by the concerted action of major metabolic groups of bacteria that break down complex organic compounds in simple substrates available to methanogen [26]: the trophic chain comprises primary fermenting bacteria (hydrolytic and acidogen), secondary fermenting bacteria (proton-reducing acetogenic bacteria), and only at least, alternatively, two functional types of methanogens (hydrogenotrophic and acetoclastic) [2, 16, 38]. Moreover another functional group co-occurred to build up the complexity of the trophic web, the homoacetogenic bacteria [42]; they connect, in reversible way, the pool of one-carbon compounds and  $\text{H}_2$  to that of acetate. So, it can be said that the energetic constrains together with the metabolic limits of the *Archaea* have pushed different kinds of microorganisms in a very efficient cooperation evolving the very complex process of the AD.

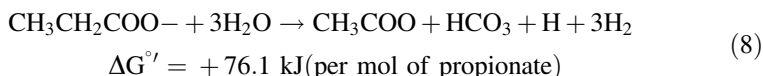
From an ecological point of view the main groups of bacteria are associated to functional guilds [62], i.e. strains of microorganisms generally taxonomical related that perform similar functions, often in a similar way. Inside each guild, single microbial strain can be replaced by another, as a consequence of ecological constrains, but every functional role must be present in order to complete the whole AD process. This means that it is not possible to achieve a complete AD process with a single bacterial strain or, conversely, even if a single kind of substrate is provided or is available, the AD process is still carried on by many microorganism strains that interact together. The cooperation relationships evolved in methanogen community are of different kind of strength and some of them evolved even in mutualistic way, and therefore obliged relationships. The most extreme case is a special symbiotic cooperation termed *syntrophism*: it refers to a kind of relationship evolved between metabolically different types of bacteria which depend on each other for substrate degradation for energetic reasons. Shink [47] underlines that the mutual dependence comprises the fact that neither partner can operate without the other and that together they exhibit a metabolic activity that neither one could accomplish on its own. This means that in natural environment the mutual dependence cannot be overcome by simply adding a co-substrate or any type of nutrient.

Relatively to AD the relationship was already identified in 1979 by McInerney et al. [39] describing the mutual cooperation of fatty acid-oxidizing fermenting bacteria or secondary fermenting bacteria, with  $\text{H}_2$ -oxidizing methanogens. During microbiological researches related to AD, it happens that some isolated culture, initially considered pure, shown to be cocultures of two partner organisms: *Archaea* populations thrive using  $\text{H}_2$  that is produced by other microorganism, the syntrophic ones, which in turn need someone else that is an  $\text{H}_2$  scavenging, i.e. the *Archaea*. *Archaea* and *Eubacteria* have coevolved their ecological niche in order to share an interspecific transfer of  $\text{H}_2$  [9], mainly to overcome thermodynamic thresholds, as we shall see below [2, 25]. More particularly, syntrophic bacteria, most belonging to *Syntrophobacter* genus from *Proteobacteria*, oxidize intermediate product during AD, like propionate or butyrate and thereby obligatorily use  $\text{H}_2$  as electron acceptor. In order to be energetically favourable, these reactions may

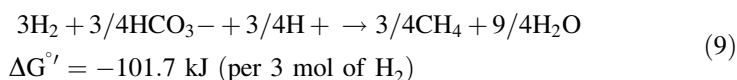
occur only when low pressure of H<sub>2</sub> is maintained, which means below 10–4 atm [16, 47].

The main thermodynamic aspects of syntrophic relationship are very well reported from [26] on their study on the bacterium strain, the syntrophic specialist *Pelotomaculum thermopropionicum*.

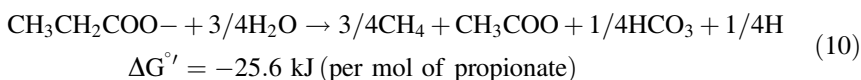
They report the example of syntrophic propionate-oxidizing bacteria that oxidize propionate into acetate, and synthesize ATP through substrate-level phosphorylation. In this reaction the reducing equivalents generated through the oxidation process are consumed by H<sub>2</sub> (or formate) production. The interesting thing is that the Gibbs's free energy change of this reaction is positive, and the reaction is unfavorable for bacteria under standard conditions.



But the reaction becomes energetically favourable, if the H<sub>2</sub> partial pressure is below 10–4 atm. In methanogenic environments, hydrogenotrophic methanogens play the role of H<sub>2</sub> subtractors, conserving energy by producing methane from H<sub>2</sub>/CO<sub>2</sub>.



When the two reactions (Eqs. 8 and 9) concomitantly occur as if it were an *unicum*, the syntrophic degradation of propionate becomes energetically feasible (10)



It is important to note that in this case the Gibbs free energy change of the overall reaction is very low, less than the energy required for the synthesis of ATP (40–70 kJ/mol of ATP). This means that syntrophic bacteria and hydrogenotrophic methanogens thrive by sharing very little energy and were forced to coevolve into an extremely efficient and complex catabolic systems enabling them to survive under such thermodynamically extreme conditions [25].

In this contest, a really interesting scientific challenge is to understand the evolutionary steps that led to the syntrophic relationships.

It is well-known that methanogen *Archaea* are among the oldest organisms on earth: because they evolved in primordial environments, at the present they are considered extremophiles, mainly in terms of temperature, pressure, composition of the atmosphere and pH value. *Archaea* produce methane using only a limited range of substrates, such as H<sub>2</sub>/CO<sub>2</sub>, formate and some methyl acetate compounds.

Probably, in ancient times, *Archaea* obtained the  $H_2$  through a “geochemical route”, nearby submarine hydrothermal vents, where  $H_2$  is produced by the serpentinization process [27, 52].

Moreover, Amend et al. [3] states that submarine hydrothermal vents are highly reactive chemical environments with far-from-equilibrium conditions are rich in gradients of redox, pH and temperature and harbouring the potential for exergonic chemical reactions.

Along the time methanogen *Archaea* coevolved with other organisms belonging to a different evolutionary domain, the *Eubacteria*, in the way to use the  $H_2$  they produce. Shifting from substrates of geochemical origin to those generated by biochemical pathway, methanogenesis became independent from the hydrothermal sites and the whole process of AD has evolved as we know it today, i.e. operated by a complex array of microorganisms in which the metabolic activities of some anticipate those of others.

It is the results of a long coevolutionary history that have metabolically linked different bacterial domains, *Eubacteria* and *Archaea*, to overcome the severity of the environmental and energetic conditions and, at the same time, have driven methanogen at the end of the AD process.

## 5 Biological Clean-up of Hydrogen Sulphide by Green Sulphur Bacteria Based Photobioreactor

The biogas produced from AD of manure and agroindustrial wastes can contain high amounts of hydrogen sulphide ( $H_2S$ ) [46].  $H_2S$  is also the principal odorous component in wastewater collection, treatment facilities and in the landfill biogas plants [12]. The  $H_2S$  amount depends on proteins and other sulphur-containing compounds that are reduced by sulphur reducer microorganisms during the decomposition of organic matters.

Even if the biogas production could contribute to the greenhouse gas reduction [1], the biogas cannot be used directly as a fuel because  $H_2S$  is very corrosive and it can cause damage to engines and gas pipelines. Furthermore, it brings to a less efficiency of energy production and sulphur dioxide ( $SO_2$ ) in the exhaust gases after combustion.

Moreover,  $H_2S$  is highly toxic. Continuous exposure at low concentrations (15–50 ppm) generally causes irritation to mucous membranes whereas at high concentrations may result in respiratory arrest. Prolonged exposures (30 min) at concentrations greater than 600 ppm can cause death [40].

Physical and chemical  $H_2S$  removal processes from biogas streams are available on an industrial scale: scrubbing, carbon adsorption, chemical and thermal oxidation [7, 49]. The Claus process is used for  $H_2S$  removal with sulphur recovery [10]. These processes are expensive due to high chemical requirements, energy and disposal costs.

A biological H<sub>2</sub>S removal method could be an eco-friendly and sustainable alternative. Chemotrophs bacteria are used in plants for biogas upgrading but they need oxygen supply and require careful control of growth condition to produce elemental sulphur instead of sulphate [56]. Among these, *Beggiatoa alba* forms characteristic encrustations on the apical part of digesters, but the clean-up process is not effective and the biogas produced needs further treatments.

Phototrophic bacteria from *Chlorobiaceae* family, indicated as green sulphur bacteria (GSB), oxidize H<sub>2</sub>S to elemental sulphur during the photosynthetic process. Among this group, *Cholorobium limicola* has demonstrated to be the most suitable for sulphide removal and satisfies the criteria for desirable applications [11, 29–31, 54–56]. It has high tolerance to H<sub>2</sub>S of which the inhibitory effect on growth starts at light saturation (100–300 μE m<sup>-2</sup> s<sup>-1</sup>) [60].

*C. limicola* lives in mud and stagnant water containing H<sub>2</sub>S where the light irradiation is very low; it is characterized by non-motile rod-shaped cells (0.7–1.1 μm) that occur singly or in aggregates and spinae have been evidenced on the cell wall.

Special structures called chlorosomes contain the light-harvesting pigments and are connected to the photosynthetic reaction centre, located in the cytoplasmic membrane, by the baseplate protein complex containing small amounts of bacteriochlorophyll (BChl) *a*. The light-harvesting pigments are: BChl *c*, *d* and *e* and the carotenoid chlorobactene. The light absorption spectrum of *C. limicola* is between 350 and 850 nm; the main peak is due to BChl *c* (745–755 nm) that represents the principal harvesting pigment [24, 53].

*C. limicola* is strictly anaerobic in presence of light and grows with CO<sub>2</sub> as sole carbon source which is fixed exclusively through the reductive tricarboxylic acid cycle that is less energy demanding than Calvin cycle used by the other phototrophs [51]. Sulphide (S<sup>2-</sup>) is used as electron donor in the first step of oxidation that produces elemental sulphur (S<sup>0</sup>) globules deposited outside the cells. The S<sup>0</sup> is further oxidized to sulphate (SO<sub>4</sub><sup>2-</sup>) in lack of sulphide and excess of light [33, 61]. The relationship between S<sup>2-</sup> loading rate, light irradiance (W m<sup>-2</sup>), S<sup>0</sup> or SO<sub>4</sub><sup>2-</sup> formation is well described by the “van Niel curve” [11].

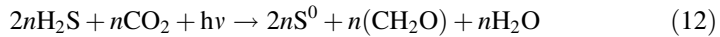
The complete oxidation of sulphide is showed in (11):



Some authors have found that S<sup>2-</sup> pulsing is a key factor in the control of the spination process that seems to be a structural adaption of the cells to special environments with highly variable S<sup>2-</sup> concentration. In fact, the number of spinae comes up with S<sup>2-</sup> underloading. These observations indicate that the presence of spinae favours the retention of the external sulphur globules physically attached to the cell wall. Therefore, sulphur globules retained near the cells could easily be oxidized to SO<sub>4</sub><sup>2-</sup> [43].

Commonly the further elemental sulphur oxidation to SO<sub>4</sub><sup>2-</sup> is considered not suitable for industrial applications, as the S<sup>0</sup> is easier to separate and recycle. Moreover, the SO<sub>4</sub><sup>2-</sup> release in the environment could constitute a threat. Therefore,

it is very important to set both a proper light intensity and a sulphide-loading rate to have an efficient  $\text{H}_2\text{S}$  removal process avoiding the formation of  $\text{SO}_4^{2-}$  (12):



So far photosynthetic bacteria have not yet reached the applicability to industrial gas cleaning-up processes; the main reason they have not been yet commercially used has to be related to the illumination costs. Most of the studies investigated bacteria growth by using common incandescent light bulbs [4] or infrared lamps [22]. Since light-emitting diodes (LED) technology came out, new possibilities to use photosynthetic bacteria with low energy demand became available. The employment of a photobioreactor equipped with LED with a wavelength peak near to the BChl *c* absorption range reported a high sulphide conversion compared to infrared or incandescent bulb light sources [29, 30, 54, 55]. However, a higher efficiency of infrared bulbs is still hypothesized [55]. These studies are carried out using liquid effluents or synthetic gas mixtures and are limited to short experimental time.

In order to consider the feasibility of a photobioreactor based on *C. limicola* culture further trials were performed with different goals:

- to analyze different light parameters (wavelength and intensity) that best fit the process;
- to test a photobioreactor directly fed with biogas produced by an AD pilot plant [15];
- to design an optimized photobioreactor focusing on best geometry allowing the light penetration through the system reaching the total removal of  $\text{H}_2\text{S}$  (patent deposit number RM2015A000082).

Future perspectives will be to scaled up to a photobioreactor operating on a large temporal scale testing the stability of bacterial inoculum.

Moreover, a simultaneous clean-up and  $\text{H}_2$  ( $\text{H}_2$ ) photoproduction process is under study by syntrophic coculture of GSB with sulphur-reducing bacteria like *Desulfuromonas acetoxidans* [66]. In these syntrophic cocultures  $\text{H}_2$  is produced as acetate is produced by a light-driven sulphur cycle where acetate is used as electron donor by the sulphur-reducing bacteria which convert sulphur to sulphide while the GSB reoxidised sulphide to sulphur. In presence of a high reducing power the photoproduction of  $\text{H}_2$  by nitrogenase comes up.

## 6 Conclusions

AD technologies are well-developed and the plant number is increasing in both industrialized and emerging countries but suboptimal biogas productions are often observed both in terms of quantity and quality. This is in part due to the fact that dynamics and interactions between microorganisms, the engine of the AD process, are not well-known.

The study of the microbial process in natural ecosystems, as well as the investigations on the potential offered by functional microbial biodiversity, can play a pivotal role for the resolution of technological issues related to the energetic valorisation of organic waste. The efficiency of biogas production can be increased if microbial ecological principles are applied, mainly to fulfil microbial requirements.

## References

1. Abraham ER, Ramachandran S, Ramalingam V (2007) Biogas: can it be an important source of energy? *Environ Sci Pollut Res* 14(1):67–71
2. 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* 2014:1–21
3. Amend JP, LaRowe DE, McCollom TM, Shock EL (2013) The energetics of organic synthesis inside and outside the cell. *Philos Trans R Soc B: Biol Sci* 368:1622
4. Ball AS, Nedwell DB, Perkins RG (2007) Oxidation of hydrogen sulphide in sour gas by *Chlorobium limicola*. *Enzyme Microb Technol* 41:702–705
5. Cai ML, Liu JX, Wei YS (2004) Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. *Environ Sci Biotechnol* 38:3195–3202
6. Casalot L, Rousset M (2001) Maturation of the [NiFe] hydrogenases. *Trends Microbiol* 9:228–237
7. Cha JM, Cha WS, Lee JH (1999) Removal of organosulphur odour compounds by *Thiobacillus novellas* SRM, sulphur-oxidizing microorganisms. *Process Biochem* 34(6–7):659–665
8. Chung YC, Huang C, Tseng CP (2001) Biological elimination of H<sub>2</sub>S and NH<sub>3</sub> from waste gases by biofilter packed with immobilized heterotrophic bacteria. *Chemosphere* 43:1043–1050
9. Conrad R (1999) Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *FEMS Microbiol Ecol* 28(3):193–202
10. Cooper DC, Alley EC (1986) Air pollution control: a design approach (Chapter 14, 411–450). PWS Engineering, Boston
11. Cork D, Mather J, Maka A, Srnak A (1985) Control of oxidative sulphur metabolism of *Chlorobium limicola* forma *thiosulfatophilum*. *Appl Environ Microbiol* 49:269–272
12. Cox HHJ, Deshusses AM (2001) Co-treatment of H<sub>2</sub>S and toluene in a biotrickling filter. *Chem Eng J* 3901:1–10
13. Das D, Veziroglu TN (2001) H<sub>2</sub> production by biological processes: a survey of literature. *Int J Hydrogen Energy* 26:13–28
14. Davila-Vazquez G, Arriaga S, Alariste-Mondragón F, Leon-Rodriguez A, Rosales-Colunga LM, Razo-Flores E (2008) Fermentative biohydrogen production: trends and perspectives. *Environ Sci Biotechnol* 7:27–45
15. De Luca E, Felici C, Corsaro N, Rosa S, Signorini A, Izzo G (2014) Hydrogen sulphide removal from gas stream by green sulphur bacteria under LED illumination. In: proceedings of the 22nd European biomass conference and exhibition, 23–26 June 2014, Hamburg, Germany
16. Demirel R, Scherer P (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. *Environ Sci Biotechnol* 7:173–190
17. Elefsiniotis P, Oldham WK (1994) Effect of HRT on acidogenic digestion of primary sludge. *J Environ Eng* 120:645–660



18. Fang HHP, Yu HQ (2000) Effect of HRT on mesophilic acidogenesis of dairy wastewater. *J Environ Eng* 126:1145–1148
19. Hallenbeck PC (2005) Fundamentals of the fermentative production of hydrogen. *Water Sci Technol* 52:21–29
20. Hallenbeck (2009) Fermentative hydrogen production: principles, progress, and prognosis. *Int J Hydrogen Energy* 34:7379–7389
21. Henry MP, Sajjad A, Ghosh S (1987) The effects of environmental factors on acid-phase digestion of sewage sludge. In: *Proceedings 42nd Purdue industrial waste conference, Indiana*, pp 727–737
22. Henshaw PF, Bewtra JK, Biswas N (1998) Hydrogen sulphide conversion to elemental sulphur in a suspended-growth continuous stirred tank reactor using *Chlorobium limicola*. *Water Res* 32(6):1769–1778
23. <http://rstb.royalsocietypublishing.org/content/368/1622/20120255.full.pdf+html>
24. Imhoff JF (1995) Taxonomy and physiology of phototrophic purple bacteria and green sulphur bacteria. Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, The Netherlands. Chap. 1, pp 1–15
25. Jackson BE, McInerney MJ (2002) Anaerobic microbial metabolism can proceed close to thermodynamic limits. *Nature* 415:454–456
26. Kato S, Watanabe K (2010) Ecological and evolutionary interactions in syntrophic methanogenic consortia. *Microbes Environ* 25(3):145–151
27. Kelley DS, Baross JA, Delaney JR (2002) Volcanoes, fluids, and life at mid-ocean ridge spreading centers. *Annu Rev Earth Planet Sci* 30:385–491
28. Khanal SK, Chen WH, Li L, Sung S (2004) Biological hydrogen production: effects of pH and intermediate products. *Int J Hydrogen Energy* 29:1123–1131
29. Kim BW, Kim EH, Chang HN (1991) Application of light emitting diodes as a light source to a photosynthetic culture of *Chlorobium thiosulfatophilum*. *Biotechnol Tech* 5(5):343–348
30. Kim JY, Kim BW, Chang HN (1996) Desulfurization in a plate type gas-lift photobioreactor using light emitting diodes. *Korean J Chem Eng* 13(6):606–611
31. Kim BW, Chang KP, Chang HN (1997) Effect of light on the microbiological desulfurization in a photobioreactor. *Bioprocess Bioeng* 17:343–348
32. Kraemer J, Bagley D (2006) Supersaturation of dissolved H<sub>2</sub> and CO<sub>2</sub> during fermentative hydrogen production with N<sub>2</sub> sparging. *Biotechnol Lett* 28:1485–1491
33. Larsen H (1952) On the culture and general physiology of the green sulphur bacteria. *J Bacteriol* 64:187–196
34. Lee YJ, Miyahara T, Noike T (2001) Effect of iron concentration on hydrogen fermentation. *Bioresour Technol* 80:227–231
35. Li D, Chen H (2007) Biological hydrogen production from steam-exploded straw by simultaneous saccharification and fermentation. *Int J Hydrogen Energy* 32:1742–1748
36. Li YF, Ren NQ, Chen Y, Zheng GX (2007) Ecological mechanism of fermentative H<sub>2</sub> production by bacteria. *Int J Hydrogen Energy* 32:755–760
37. Liu G, Shen J (2004) Effects of culture and medium conditions on hydrogen. *J Biosci Bioeng* 98(4):251–256
38. Liu Y, Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann New York Acad Sci* 1125:171–189
39. McInerney MJ, Bryant MP, Pfennig N (1979) Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch Microbiol* 122:129–135
40. MSDS (1996) Material safety data sheet for hydrogen sulphide. Murray Hill, NJ: BOC Gases. <http://www.vngas.com/pdf/g94.pdf>. Accessed 20 Feb 2006
41. Nath K, Das D (2004) Improvement of fermentative hydrogen production: various approaches. *Appl Microbiol Biotechnol* 65:520–529
42. Noory M, Saady Cata (2013) Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unresolved challenge. *Int J Hydrogen Energy* 38:13172–13191

43. Pibernat IV, Abella CA (1996) Sulfide pulsing as the controlling factor of spinae production in *Chlorobium limicola* strain UDG 6038. *Arch Microbiol* 165:272–278
44. Redwood MD, Paterson-Beedle M, Macaskie LE (2009) Integrating dark and light bio-hydrogen production strategies: towards the hydrogen economy. *Environ Sci Biotechnol* 8:149–185
45. Ruggeri B, Zitella P, Goretti M, Scaletta A (2008) hydrogen da fermentazione di biomasse con popolazioni batteriche miste. *La Termotecnica*
46. Schieder D, Quicker P, Schneider R, Winter H, Prechtl S, Faulstich M (2003) Microbiological removal of hydrogen sulphide from biogas by means of a separate biofilter system: experience with technical operation. *Water Sci Technol* 48(4):209–212
47. Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* 61(2):262–280
48. Schlesinger WH (1991) Biogeochemistry. An analysis of global change. Academic Press, Inc., San Diego. ISBN 0-12-625157-6
49. Shareefdeen Z, Herner B, Wilson S (2002) Biofiltration of nuisance sulphur gaseous odors from a meat rendering plant. *J Chem Technol Biotechnol* 77:1296–1299
50. Sinha P, Pandey A (2011) An evaluative report and challenges for fermentative biohydrogen production. *Int J Hydrogen Energy* 36:7460–7478
51. Sirevåg R (1995) Carbon metabolism in green bacteria. In: Blankenship RE, Madigan MT, Bauer CE (eds) Anoxygenic photosynthetic bacteria. The Netherlands, pp 871–883
52. Sousa FL, Thiergart T, Landan G, Nelson-Sathi S, Pereira IAC, Allen JF, Lane N, Martin WF (2013) Early bioenergetic evolution. *Philos Trans R Soc B: Biol Sci* 368:1622
53. Stanier RY, Ingraham JL, Wheelis ML, Painter PR (1986) The microbial world, 5th edn. Prentice-Hall Inc, Englewood Cliffs
54. Syed MA, Henshaw PF (2003) Effect of tube size on performance of a fixed-film tubular bioreactor for conversion of hydrogen sulphide to elemental sulphur. *Water Res* 37(8):1932–1938
55. Syed MA, Henshaw PF (2005) Light emitting diodes and an infrared bulb as light sources of a fixed-film tubular photobioreactor for conversion of hydrogensulphide to elemental sulphur. *J Chem Technol Biotechnol* 80:119–123
56. Syed MA, Henshaw PF (2006) Modelling of a fixed-film tubular photobioreactor for conversion of hydrogen sulphide to elemental sulphur. *Indian J Chem Technol* 13:226–232
57. Syed MA, Soreanu G, Falletta P, Béland M (2006) Removal of hydrogen sulphide from gas streams using biological processes—a review
58. Tricase C, Lombardi M (2009) State of the art and prospects of Italian biogas production from animal sewage: technical-economic considerations. *Renew Energy* 34:477–485
59. Trüper HG, Schlegel HG (1964) Sulphur metabolism in Thiorhodaceae, I. Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie Van Leeuwenhoek* 30:225–238
60. Van Gernerden H (1984) The sulphide affinity of phototrophic bacteria in relation to the location of elemental sulphur. *Arch Microbiol* 139:289–294
61. Van Niel CB (1931) On the morphology and physiology of the purple and green sulphur bacteria. *Archiv fur Mikrobiologie* 3:1–112
62. Vanwonterghem I, Jensen PD, Dennis PG, Hugenholtz P, Rabaey K, Tyson GW (2014) Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. *Int Soc Microbiol Ecol* 8:2015–2028
63. Volume 2014, Article ID 183752. <http://dx.doi.org/10.1155/2014/183752>
64. Wang J, Wan W (2008) Effect of Fe<sub>2+</sub> concentration on fermentative hydrogen production by mixed cultures. *Int J Hydrogen Energy* 33:1215–1220
65. Wang J, Wan W (2008) Effect of Ni<sub>2+</sub> concentration on biohydrogen production. *Bioresour Technol* 99:8864–8868

66. Warthmann R, Cypionka H, Pfennig N (1992) Photoproduction of H<sub>2</sub> from acetate by syntrophic cocultures of green sulphur bacteria and sulphur reducing bacteria. *Arch Microbiol* 157:343–348
67. Wei-Chih L, Yu-Pei C, Ching-Ping T (2013) Pilot-scale chemical–biological system for efficient H<sub>2</sub>S removal from biogas. *Bioresour Technol* 135:283–291
68. Zhang ML, Fan Y, Xing Y, Pan C, Zhang G, Lay I (2007) Enhanced biohydrogen production from cornstalk wastes with acidification pretreatment by mixed anaerobic cultures. *Int J Hydrogen Energy* 31:250–254