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

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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_2). The content of CH_4 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_2 production is due to an incomplete digestion process stopped before the methanogenic phase. The methanogenesis, in fact, consumes the amount of H_2 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_2 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_2 and CO_2. The next two stages of AD (acetogenesis–methanogenesis) lead to the production of biogas, a mixture of 50–70 % CH_4 and 50–30 % for CO_2.

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. NAD^+ oxidaze form). The NADH cannot unload its electrons in the transport chain; therefore to regenerate the pool of NAD^+ necessary for the continuation of the process, the reoxidation of NADH occurs at the expense of an intermediate compounds of the process, which acts as acceptor and the fermentation process is therefore a redox balanced in its interior.

When an organism degrade,s 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 H_2. Generally the production of 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 H^+ is catalyzed by the enzyme H_2ase. 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 H_2 has the task of maintaining the redox balance, if the final products are more reduced, they result in a lower production of H_2. For example, when the ethanol, more reduced than acetate, is produced then 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, CO_2 and 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 CH_4 is the conclusion of the AD process. Its production can take place essentially through two different pathways of reactions: a path provides CH_4 by hydrogenotrophic bacteria that operate under the anaerobic oxidation of 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 CH_4 production occurs through this second pathway. The quantity and composition of the biogas (at least in terms of CH_4 and 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 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 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 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_2 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_2 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_2 production, the alternative possible solutions are:

(a) photo-bioreactors in which purple non-sulphur bacteria produce H_2 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_2.

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_2 [14]:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2 \quad (1)$$

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_2/moles substrate) varies depending of the final products that are formed, in fact, as previously mentioned, the H_2 is produced exclusively to balance the redox reaction, when the substrate is more or less reduced the formation of H_2 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_2.

$$C_6H_{12}O_6 + 2H_2O + 2CO_2 + 4H_2 \rightarrow 2CH_3COOH \text{ acetic} \quad (2)$$

$$CH_3COCH_3 + H_2O \rightarrow C_6H_{12}O_6 + 3CO_2 + 4H_2 \text{ acetone} \quad (3)$$

$$CH_3CH_2CH_2COOH \rightarrow C_6H_{12}O_6 + 2CO_2 + 2H_2 \text{ butyric} \quad (4)$$

H_2 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_2 and ensures a potential productivity of 4 mol of H_2 per mole of glucose, while the butyric reaches a maximum of 2 mol.

$$HCOOH \rightarrow H_2 + CO_2 \text{ decomposition of formic} \quad (5)$$

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_2 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^+ 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_2) is prevented, but below pH 5 the productivity of H_2 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_2 yield is lower with a high initial pH: in fact it causes a rapid production of H_2 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_2 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_2 production is supported by mesophilic conditions and also in some thermophilic.

The effect of temperature on H_2 production can be explained thermodynamically by Van't Hoff equation

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

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

$$\begin{aligned} &C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \\ &\Delta G^{\circ} = -176.1 \text{ kJ/mol} \\ &\Delta H^{\circ} = +90.69 \text{ kJ/mol} \end{aligned} \tag{7}$$

With increasing temperature the equilibrium constant kinetic increases, because the reaction is endothermic and thereby increases the concentration of H_2.

In other studies it has been reported that the volumetric rate of H_2 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_2 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_2 partial pressure inhibits the same yield of H_2 production [20]. The H_2 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_2 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_2. 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_2 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_2, 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_2, and its most reduced states CH_4 [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_2/CO_2, 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 CH_4 and 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 (hydrolitic 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 homoagetogenic bacteria [42]; they connect, in reversible way, the pool of one-carbon compounds and 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 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 H_2 that is produced by other microorganism, the syntrophic ones, which in turn need someone else that is an H_2 scavenging, i.e. the *Archaea*. *Archaea* and *Eubacteria* have coevolved their ecological niche in order to share an interspecific transfer of H_2 [9], mainly to overcome thermodynamic thresholds, as we shall see below [2, 25]. More particulary, syntrophic bacteria, most belonging to *Syntrophobacter* genus from *Proteobacteria*, oxidize intermediate product during AD, like propionate or butyrate and thereby obligatorily use H_2 as electron acceptor. In order to be energetically favourable, these reactions may

occur only when low pressure of H_2 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_2 (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.

$$\begin{gathered} CH_3CH_2COO- + 3H_2O \rightarrow CH_3COO + HCO_3 + H + 3H_2 \\ \Delta G^{\circ\prime} = +76.1 \text{ kJ(per mol of propionate)} \end{gathered} \tag{8}$$

But the reaction becomes energetically favourable, if the H_2 partial pressure is below 10–4 atm. In methanogenic environments, hydrogenotrophic methanogens play the role of H_2 subtractors, conserving energy by producing methane from H_2/CO_2.

$$\begin{gathered} 3H_2 + 3/4HCO_3- + 3/4H+ \rightarrow 3/4CH_4 + 9/4H_2O \\ \Delta G^{\circ\prime} = -101.7 \text{ kJ (per 3 mol of } H_2) \end{gathered} \tag{9}$$

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)

$$\begin{gathered} CH_3CH_2COO- + 3/4H_2O \rightarrow 3/4CH_4 + CH_3COO + 1/4HCO_3 + 1/4H \\ \Delta G^{\circ\prime} = -25.6 \text{ kJ (per mol of propionate)} \end{gathered} \tag{10}$$

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_2/CO_2, 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_2S 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_2S 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_2S of which the inhibitory effect on growth starts at light saturation (100–300 μE m^{-2} s^{-1}) [60].

C. limicola lives in mud and stagnant water containing H_2S 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_2 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^{2-}) is used as electron donor in the first step of oxidation that produces elemental sulphur (S^0) globules deposited outside the cells. The S^0 is further oxidized to sulphate (SO_4^{2-}) in lack of sulphide and excess of light [33, 61]. The relationship between S^{2-} loading rate, light irradiance (W m^{-2}), S^0 or SO_4^{2-} formation is well described by the "van Niel curve" [11].

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

$$n\mathrm{H_2S} + 2n\mathrm{CO_2} + 2n\mathrm{H_2O} + \mathrm{h}v \rightarrow n\mathrm{SO_4^{2-}} + 2n\mathrm{H^+} + 2n(\mathrm{CH_2O}) \qquad (11)$$

Some authors have found that S^{2-} 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^{2-} concentration. In fact, the number of spinae comes up with S^{2-} 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_4^{2} [43].

Commonly the further elemental sulphur oxidation to SO_4^{2-} is considered not suitable for industrial applications, as the S^0 is easier to separate and recycle. Moreover, the SO_4^{2-} 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 H_2S removal process avoiding the formation of SO_4^{2-} (12):

$$2nH_2S + nCO_2 + h\nu \rightarrow 2nS^0 + n(CH_2O) + nH_2O \quad (12)$$

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 H_2S (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 H_2 (H_2) photoproduction process is under study by syntrophic coculture of GSB with sulphur-reducing bacteria like *Desulfuromonas acetoxidans* [66]. In these syntrophic cocultures 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 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.

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