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Biohydrogen production from agricultural and livestock residues within an integrated bioenergy concept

Ph.D. Thesis

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Abstract

Concerns about energy security, fossil fuel prices, and climate change issues, are leading to increasing renewable energy demand. Hydrogen is considered as one of the main possible energy carriers in future, due to its environmental (it can be converted to energy with the solely emission of water) and energetic (energy content of 120 MJ/kg, three times higher of the gasoline content of 44 MJ/kg) unique properties.

If hydrogen is currently being produced mainly by fossil sources, its production from renewable sources answers to the demand of more environment-friendly exploiting alternatives, possibly leading to a renewable-based hydrogen economy. Biomasses are an important renewable source ranging from energy-dedicated crops to livestock waste effluents, agro-industrial wastewaters, food-processing industry residues and organic fractions of the municipal solid waste (OFMSW). Thus the agricultural sector may acquire a renewed importance in the mid-term as a producer of energy sources for renewable biohydrogen production.

Among the biological ways to exploit biomasses for hydrogen production, this thesis focused its interest on anaerobic dark fermentation, which can simultaneously guarantee the production of an high-value product (H_2) at high evolution rates and the treatment of wastes, thus transformed from an environmental pollution and greenhouse gases emissions source into a valuable resource. If on the one hand this process has lots in common with anaerobic digestion, which already is a well-established technology for treating different biomass types in real-scale plants, on the other hand it is a relatively new approach, which needs to be further studied for improving its performances and being concretely applicable.

As a matter of fact, the main disadvantage of dark fermentation is its relatively low yield, compared to other bio-hydrogen production methods, which typically are between 2.4 and 3 mol H_2/mol glucose. This represents just the 20-25% of the 12 mol of H_2 theoretically obtainable by glucose fermentation. Therefore, generally two different (but not mutually exclusive) options could be chosen for improving the process and making it ready for full-scale applications: the optimization of the biological, biochemical, chemical-physical operative parameters that regulate process; or the coupling of this bioprocess with other technologies capable of exploiting the organic matter not fully used by the dark fermentative approach. For example, Microbial Electrolysis Cells (MECs) are able to biologically oxidize the organic matter (from simple substrates like volatile

fatty acids, lactic acid, glucose, cellulose, to actual wastewaters) releasing electrons from an anode to a cathode where potentially pure hydrogen can be formed from protons in the water.

Papers I and II basically belong to the first strategy. In Paper I indeed, two waste biomasses were co-digested: in consideration that in the Po Valley area (Italy) swine manures (SM) are yearly produced at high waste density levels and could be a cause of environmental problems, this waste was used as a co-substrate for biohydrogen production by the thermophilic fermentation of easily degradable and carbohydrate-rich materials, such as fruit and vegetable market waste (FVMW). Biohydrogen production rates and process stability were thus simultaneously maximized, thanks to the endogenous buffer capacity of manure, through the combination of a suitable composition (as FVMW/SM) of the feeding material and the hydraulic retention time (HRT) of the process. Thus, livestock manure represented not only a renewable source for supplying the production of biohydrogen, but also a source of alkali to be used for avoiding the addition of exogenous chemicals (alkali) to maintain the pH, and so the metabolic pathways and bacterial communities, into an optimal domain for biohydrogen production.

To further study and optimize the bio-H_2 production in laboratory-scale processes, but also to find applicable tools for favoring dark fermentation application in full-scale biogas plants, Paper II succeeded in obtaining mixed microbial cultures from natural sources (soil-inocula and anaerobically digested materials) which reached high hydrogen yields with glucose and were used to explore the potential of bio-hydrogen production from four organic substrates of possible interest for full-scale plants (market bio-wastes, maize silage, swine manure, OFMSW). In direct prosecution of the positive co-digestion results shown in Paper I and looking for future transfer of this bioprocess technical solutions to full-scale systems, Paper II used the enriched mixed microflora for evaluating the co-fermentation of a mixture of OFMSW and swine manure in a lab-scale continuously-fed CSTR (continuously stirred tank reactor) digester. Despite the good results obtained, our study suggested that further efforts are needed for future applications of effective biohydrogen fermentation in full-scale plants.

Paper III and IV are more focused on the second scientific strategy. Paper III joins the interest toward implementation of bio-H_2 in full-scale plants and the strategy of improving the overall recovery of the energy contained in the biomass associating hydrogen production to other bioprocesses. Many authors report that the two-stage anaerobic digestion (AD) process, if

compared to traditional and extensively real-scale applied single-stage AD, has also other advantages, such as differentiating the biofuel production (bio-hydrogen and bio-methane), potentially reducing the plant dimensions and costs, improving the overall biogas production yields and allowing higher CH_4 concentrations in the biogas produced in the second stage, thus decreasing the biogas purification costs. Therefore, a two-stage laboratory-scale CSTR anaerobic digester, fed with a mixture of agricultural and livestock residues, was monitored for a long run (approximately 700 hours) and compared to a similar one-stage reactor. This study obtained a good hydrogen yield per kg of biomass treated and partially confirmed the advantages previously illustrated, even if it reached almost the same overall energy recovery of the single stage process.

Aiming at other possible biological strategies to improve the energy and hydrogen recovery efficiency with the use of effluents from a first dark fermentative stage, a relatively new electrohydrogenesis device (MEC) was studied. Paper IV explores the rate and the yield of biogas (a mixture of H_2, CH_4 and CO_2) produced by MEC exploiting an actual industrial wastewater with high methanol content, a compound never before reported to be used in a MEC device. The energetic recovery and treatment performance of the process was evaluated and also compared with a simulation of anaerobic digestion of the same wastewater, revealing the economical competitiveness of the MEC technology with the AD process. This leads to future research perspectives aiming to realize a laboratory-scale two-stage reactor with a MEC using the volatile-rich effluent of a first dark fermentative stage.

Keywords: Bio-hydrogen, biomasses, MEC, biorefinery

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List of International Refereed Papers and Congress Communications

International Refereed Papers

This thesis is based on the work contained in the following papers, referred to by their Roman numerals in the text:

I. Tenca A., Schievano A., Perazzolo F., Adani F., Oberti R. 2011. Biohydrogen from thermophilic co-fermentation of swine manure with fruit and vegetable waste: Maximizing stable production without pH control. *Bioresource Technology* 102(18): 8582-8588.

II. Tenca A., Schievano A., Lonati S., Malagutti L., Oberti R., Adani F. 2011. Looking for practical tools to achieve next-future applicability of dark fermentation to produce bio-hydrogen from organic materials in Continuously Stirred Tank Reactors. *Bioresource Technology* 102(17): 7910-7916.

III. Tenca A., Schievano A., Scaglia B., Oberti R., Adani F. Two-stage vs. single-stage fermentation process: comparison of energetic performances and chemical characterization. (To be submitted to *International Journal of Hydrogen Energy*)

IV. Tenca A., Cusick R., Logan B.E. MEC and Anaerobic Digestion performance comparison with complex industrial wastewater. (To be submitted to *Biotechnology and Bioengineering*)

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Congress Communications

I. Schievano A., Consonni E., Tenca A., Oberti R., Adani F. 2009. Bio-hydrogen production from bio-waste: ready for full-scale applications? (*Ecomondo 2009* Proceedings)

II. Schievano A., Tenca A., Oberti R., Adani F. 2009. An operational strategy to produce biohydrogen: the use of digestate for process control (*Hypothesis VIII* Proceedings)

Chapter 1

Hydrogen energy infrastructure and biological production of hydrogen

1.1 Energy and environmental issues: the factors favoring the hydrogen energy

The increasing global demand for finite oil and natural gas reserves together with the national energy security need, are driving the scientific community into the search for alternatives to fossil fuels. During the 20th century, the industrial and societal development was driven by and depended on the fossil resources and related technologies, but with the rapidly expanding world population and the increase of prosperity in less developed countries, it is expected that the world consumption of energy and resources will increase with a minimum factor of 3 by the year 2050 (Boeriu *et al.*, 2005). Stating that fossil fuel reserves are a diminishing raw material and that the fossil resources are non-uniformly distributed, Rifkin argues

that global oil production will fail to meet this increasing demand for energy in the next 10-20 years (Rifkin, 2002). Even if oil and fossil fuel production wouldn't reach an international crisis, many nations of the world are already and increasingly adopting alternatives to fossil fuels, taking increased carbon dioxide issues seriously and implementing measures to reduce greenhouse gas emissions. Indeed, fossil fuels possess undoubtedly very useful properties not shared by non-conventional energy sources, but in addition to being not renewable, they are not environmentally friendly. The pollutants emitted by fossil energy systems (e.g. CO, CO_2, C_nH_m, SO_x, NO_x, radioactivity, heavy metals, ashes, etc.) are greater and more damaging than those that might be produced by a renewable based energy system (Momirlan and Veziroglu, 2002). Although there is disagreement about the specific effects of greenhouses gases on global temperature, it is a fact that the concentration of atmospheric CO_2 have increased about 30% in the past 150 years and that the fossil resources are the major source of this additional CO_2 (Chamberlain *et al.*, 1982).

Therefore the world is striving for the search of a newer, cleaner and renewable energy source that can be easily transported, used in vehicles and that in principle will never run out. Hydraulic, solar, wind, tidal, geothermal energy and also energy from renewable raw materials such as biomass, are all expected to significantly contribute in the mid- to long-term to the energy demand(Soetaert and Vandamme, 2005).

Within this scenario, hydrogen has demonstrated a big potential to both reduce the dependence on oil and to lower the greenhouses gas emissions. Hydrogen was reported to be the "fuel of future" since the energy crisis of the '70s, and its popularity as a fuel source has always followed the crisis coming from the extensive use of nonrenewable fuels. As a matter of fact, after great efforts put into hydrogen research during the early '70s, like other alternative energy

technologies hydrogen lost its importance with the drop of the oil price at the end of the crisis. But again, with the emerging concerns about the greenhouse effect in the '90s, a new crisis reignited mainstream interest in post-fossil fuel hydrogen-based economy (Benemann, 1996).

Although H_2 is an energy carrier rather than a traditional fuel found in and harvested from nature, it joins the advantages to be a mobile source of energy and to be environmentally friendly (especially if manufactured by capturing energy from renewable starting materials) (Logan, 2004; Veziroglu and Barbir, 1992). Nowadays, the "Global Hydrogen Vision" envisions hydrogen as a flexible, safe, affordable, domestic energy resource to be used in all sectors of the economy, in a wide variety of applications, including fuel for automobiles and in all regions of the world. Since it can be used either as the fuel for direct combustion in an internal combustion engine or as the fuel for a fuel cell, hydrogen could join electricity as a primary energy carrier and provide the foundation for a globally sustainable energy system (Kotay and Das, 2008).

H_2 has the highest energy content per unit weight among the known gaseous fuels (143 GJ ton^{-1}, which is 2.75 times greater than hydrocarbon fuels) and at the same time it is a carbon-free fuel which oxidizes to water as a combustion product (Armor, 2005). The only pollutant that eventually can arise is the nitrogen oxide that comes from the combination of the oxygen and nitrogen in the air. This occurs only if the hydrogen is not recombined with pure oxygen, that is using air as an oxidant, and with high reaction temperatures (Momirlan and Veziroglu, 2002). Therefore hydrogen energy system could be presented as a carbon-free, natural close cycle where the water generated from its combustion becomes, together with renewable primary energy for splitting it, a source of clean and abundant energy (Fig. 1.1).

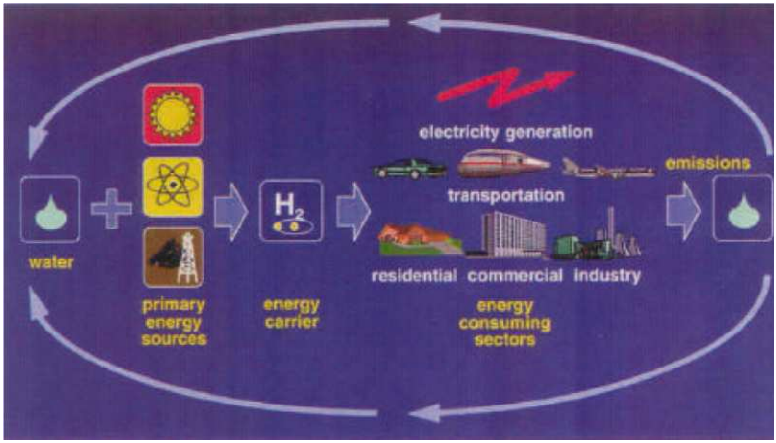

Fig. 1.1 Hydrogen energy system (Source: International Association of Hydrogen Energy, USA; Courtesy of Nath and Das, 2003).

However, independently of the source of hydrogen, there are many logistical and market challenges that must be overcome before a "hydrogen economy" can become a reality. Among them:

- *Production technologies*

Although hydrogen is the most abundant element in the universe, it must be produced from other hydrogen-containing compounds, such as fossil fuels, biomass, or water, and each method of production requires a source of energy, i.e. thermal (heat), electrolytic (electricity), or photolytic (light) energy (Kotay and Das, 2008). Currently, the hydrogen production depends mostly on natural gas and therefore is highly energy intensive and not environmental-friendly. Nearly 90% of hydrogen is obtained by steam reforming of naphtha or natural gas and by gasification of coal or by electrolysis of water (Nath and Das, 2003). For sure these strategies, especially in the case of steam reforming of methane (SRM) or other hydrocarbons (SRH) and of non-catalytic partial oxidation of fossil fuels (POX), have reached maturity for commercial exploitation but are really energy-intensive processes, requiring high temperatures

(>850 °C) (Momirlan and Veziroglu, 2002). In consideration of both energy security and environmental issues related to fossil-fuel reserves exploitation, production of hydrogen by renewable sources (such as biomasses) seems to be imperative (in-depth examination in chapter 1.3.1)

- *Hydrogen market and the required infrastructures*

If nowadays technologies challenges are firstly addressed to lowering the cost of hydrogen production, also hydrogen delivery, storage and conversion are required to be elaborated. Indeed, contrarily to what is commonly thought, demand of hydrogen is not limited to its utilization as a source of energy. Hydrogen gas is a widely used feedstock for the production of chemicals and electronic devices, the hydrogenation of fats and oils in food industry, the steel processing and the desulfurization and re-formulation of gasoline in refineries. H_2 can acts to saturate compounds, crack hydrocarbons or remove sulphur, nitrogen compounds and traces of oxygen (thus preventing the oxidative corrosion thanks to its oxygen scavenging property).

Such an amount of end-use applications makes a 50 million tons of hydrogen annually trading worldwide and is pushing the contribution of hydrogen to total energy market up to 8-10% by 2025 (United States National Hydrogen Program esteem) (Kapdan and Kargi, 2006).

Among the technologies for hydrogen storage and transport, there are four technologies available today to store hydrogen aboard vehicles (Hynek *et al.*, 1994):

- liquefied hydrogen (used by NASA and considered for airliners);
- metal hydrides (used for example by Mazda and by Daimler-Benz in passenger cars);
- compressed hydrogen gas (used on urban transport bus built by Ballard);
- carbon sorption (yet to be used on vehicles).

Moreover in recent years, new technologies have been developed as reported by an interesting state of the art review by Momirlan and Veziroglu (2002):

- new carbon variants: graphitic nanofibres and (carbon) nanotubes as hydrogen storage materials;
- new hydrogen storage alloys: La/Ce mixture, Mg or Mg_2Ni (nanocrystalline) system, nanocrystalline Zr-based AB_2 alloys;
- liquid-film type catalytic decalin dehydrogeno-aromatization for mobile storage of hydrogen;
- innovative hydrogen densification in a two stage metal hydride system.

- *Upgrading requirements*

Especially dealing with the biological processes, hydrogen produced is mostly found together with other different gaseous impurities like O_2, CO, CO_2, CH_4 and some amount of moisture. The presence of these gases lowers the heating value of hydrogen, in addition to posing some problems in efficient burning of fuels (see section 1.2). Therefore, as proposed by Nath and Das (2003) the following upgrading procedures may be used:

- CO_2 acts as a fire extinguisher and it is sparingly soluble in water: it can be separated by scrubbers or absorbed by a fifty per cent (w/v) KOH solution or monoethanolamine;

- O_2 in the gas may cause a fire hazard: it can be absorbed by an alkaline pyrogallol solution;

- Moisture in the gas mixture reduce the heating value of hydrogen: passing the gas mixture either through a dryer or a chilling unit (by condensing vapor in the form of water) is the more practical solution.

1.2 Technology for hydrogen energy use

The widespread use of hydrogen as an energy carrier will depend significantly on the availability of efficient, clean and economic

techniques for its utilization and conversion to electricity/heat. Among the main technologies for hydrogen utilization as a fuel, it is possible to distinguish internal combustion engines (ICE) from the fuel cell (FC) technologies. If the first is widely known and used, regardless of hydrogen use, the latter is now emerging as a leading technology able to replace the more polluting ICEs both in vehicle and stationary distributed energy applications and it will need deeper introduction.

1.2.1 Internal Combustion Engines

An internal combustion engine is an engine in which the combustion of a fuel (from fossil fuels - petroleum and carbon - to biofuels, vegoils and hydrogen) occurs with an oxidizer (usually air) in a combustion chamber. In an ICE, the expansion of the high-temperature and high-pressure gases produced by combustion applies direct force to some component of the engine (such as pistons, turbine blades, nozzle), thus generating useful mechanical energy.
A large number of different designs for ICEs have been developed and built, each one with different points of strength and weakness, and even if there are many stationary applications, the real strength of internal combustion engines is in mobile/automotive applications. Gasoline, Diesel, Wankel engines and open gas turbines are all examples of internal combustion engines.
Hydrogen could eventually replace conventional fossil fuels in traditional ICE. Apart for its heating volume, other key properties of hydrogen that are relevant to its employment as an engine fuel (especially for transport processes) are its remarkably high values of thermal conductivity and diffusion coefficient, in comparison to those of gasoline fuel (hydrogen: thermal conductivity at 300 K: 182.0 mW/m K, diffusion coefficient into air at NTP: 0.61 cm^2/s; gasoline: 11.2 mW/m K and 0.05 cm^2/s, respectively) (Yamin, 2006).

So according to Yamin et al. (2000), hydrogen employment in ICEs has the following advantages over gasoline:

1. Reduced deposits due to more homogeneous mixture formation.
2. Reduced engine oil dilution and increased oil life.
3. Reduced engine wear, hence increased engine life.
4. Higher compression ratios can be used which may solve the problem of reduced power output due to reduction in volumetric efficiency.
5. Elimination of emissions of CO and HC.
6. Increased fuel economy due to possible operation at leaner mixtures.

Moreover, hydrogen-fueled internal combustion engines, which already have typical efficiency of 30% (and maximum of 45%) could help in quickly filling the gap between the existing hydrocarbon-fueled ICE and future hydrogen fuel-cell technology.

On the other side, hydrogen use in ICE has some problems.

The first is its introduction into the engine, because of hydrogen wide flammability range, low energy density by volume and high flame speed (U.S. D.o.E., 2001).

The second is that its use in ICE (as in catalytic burners) may produce nitrogen oxides (NOx) emissions (Zurawski *et al.*, 2005) due to the high temperatures generated within the combustion chamber. Indeed, H_2 produces only water if combusted with oxygen, following the reaction

$$H_2 + 1/2\ O_2 \rightarrow H_2O$$

but also NOx are produced if it is combusted with air as follows

$$H_2 + O_2 + N_2 \rightarrow H_2O + N_2 + NOx$$

So, depending on the operating strategy used (i.e. the air/fuel ratio, the engine speed and compression ratio, the ignition timing, etc.), an hydrogen engine can produce from almost zero emissions to high NOx and even carbon monoxide emissions.

Alternative strategies to the use of pure hydrogen in ICE are also possible. For example H_2 can be used as supplementary fuel to enhance the combustion properties of natural gas (a commercially available mixture known as Hythane - i.e. 20% hydrogen and 80% natural gas -), without requiring modifications to a natural gas engine: both gases can be stored in the same tank, and emissions reduction by more than 20% are possible (U.S. D.o.E., 2001). Indeed, the hydrogen presence allows lower combustion temperatures, simultaneously leading to lower NOx emissions and enhancing the combustion process with recovery of the power and energy consumption penalties associated with natural gas.

1.2.2 Fuel cells

A fuel cell (FC) is an electrochemical device that converts the chemical energy of gaseous (e.g. hydrogen, natural gas, and biomass derived gas) or solid (mainly coal) fuels directly into electrical energy (and heat) via an electrochemical process with high conversion efficiency (Edwards *et al.*, 2007). Differently from a battery, which is an energy storage device, a fuel cell is an energy conversion device that can produce electricity as long as the fuel and the oxidant are provided to the electrodes and that doesn't run out (unless undesired events, such as component degradation/corrosion). The concept of the FC was firstly developed by W.R. Grove in 1839, who successfully produced an electric current and water by combining hydrogen gas with oxygen.
The basic FC consists of an electrolyte layer in contact with a porous anode and cathode on either side. A fuel, such as hydrogen, is fed to the anode where negatively charged electrons are catalytically separated from positively charged ions. From the anode and through an electrolyte a ionic current flows toward the cathode where protons combine with oxygen or air, resulting in water production.

Simultaneously, the excess electrons flows through an external electric circuit, generating an electric current (Edwards *et al.*, 2007). Depending on the FC type and its conversion efficiency, different amount of the energy developed by the hydrogen oxidation reaction (enthalpy of 285.8 kJ/mol under standard conditions) are converted into electricity, the remaining into heat. Because fuel cells are not subject to the intrinsic limitations of the Carnot cycle, they convert fuel into electricity at more than double the efficiency of internal combustion engines. In transportation, hydrogen fuel cell engines operate at an efficiency of up to 65%, compared to 25-30% for current oil-fueled car engines. As the reaction at the basis of the FC is an exothermic one, when heat generated in fuel cells is also used in combined heat and power (CHP) systems, an extremely high overall efficiency of 85% or more can be achieved (Dutton, 2002). Moreover, unlike internal combustion engines, fuel cells demonstrate high efficiency across most of their output power range. This scalability makes them ideal for a variety of applications from mobile phone batteries through vehicle applications to large-scale centralized or decentralized stationary power generation.
Even from the environmental point of view the fuel cells seem to be better than ICEs because, operating at lower temperature, only water and virtually no pollutant (NOx) are released. If the hydrogen fuel could be sourced from renewable routes and not be hydrocarbon-based, real zero emission will be reached by hydrogen-powered fuel cell vehicles (HFCV). Even if compared with other zero-emission vehicles (as battery-driven electric cars) HFCV may relay on a technology (FC) characterized by a much longer operational lifetime and providing the same high specific energy as traditional combustion engines (Winter and Brodd, 2004).

Several types of fuel cells operating on a variety of fuels and suitable for different energy applications have been developed, so they are

generally categorized by low (LT) or high (HT) operation temperature.

Alternatively, if classified according to the material used as the electrolyte we can distinguish:

- alkaline fuel cells (AFC) (both LT and HT);
- phosphoric acid fuel cell (PAFC) (LT);
- proton exchange membrane fuel cell (PEMFC) (LT);
- molten carbonate fuel cell (MCFC) (HT);
- solid oxide fuel cell (SOFC) (HT).

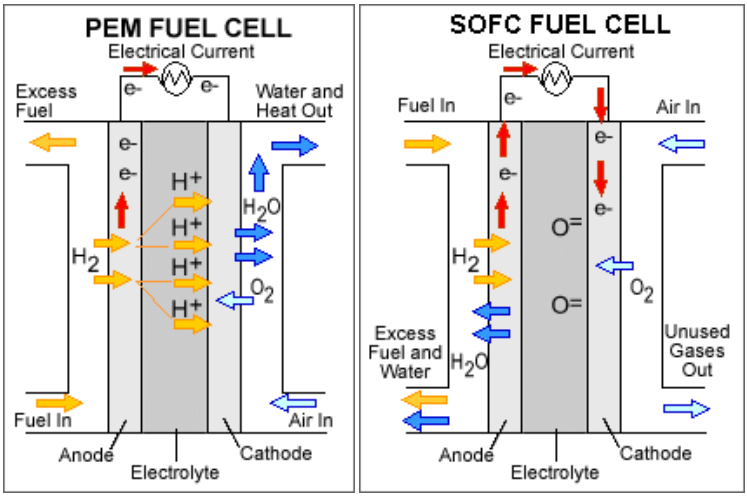

Fig. 1.2 Schematic of a PEM fuel cell (**left**) and a SOFC fuel cell (**right**).

- Low-temperature fuel cells

The most interesting technology among the low-temperature FCs is the PEMFC (Figure 1.2), which means Polymer Electrolyte Membrane or Proton Exchange Membrane Fuel Cell.

Its particular architecture uses a thin solid polymer as an electrolyte (typically Nafion kept moist using liquid water) and porous carbon electrodes containing a platinum catalyst. Typically they are fueled with high purity hydrogen and operate at relatively low temperatures,

around 80 °C, which positively affects the components used, resulting in better durability.
In details in a PEMFC:
1. Hydrogen gas is pressurized and channeled through flow field plates and throughout the anode gas diffusion electrode.
2. The catalyst layer (usually platinum powder mounted on very thin carbon paper) between the anode and the PEM electrolyte accelerates the separation of the hydrogen gas into two negatively charged free electrons and two positively charged hydrogen proton ions.
3. The positively charged hydrogen protons travel through the moist PEM membrane to the cathode while the free electrons travel through the external load to the cathode.
5. Oxygen gas (or normal air) is pressurized and channeled throughout the cathode gas diffusion electrode.
6. The catalyst layer between the cathode and the PEM accelerates the separation of the oxygen gas into two oxygen molecules.
7. Hydrogen protons, free electrons and oxygen molecules combine inside the cathode to form a water molecule (H_2O) while releasing heat (then used outside of the FC or just exhausted).

A typical PEM running at 0.6 to 0.8 volts has an efficiency (conversion of hydrogen into electricity) of about 50 ± 10%, which is much better than 20% efficiency of common gasoline engines. PEMFC stacks (a set of connected cells) reach high-power density and have low weight and volume, compared with other FC, therefore they are particularly suitable for the scalability of this technology. Indeed, PEM fuel cells are very close to mass production and are primarily used for automotive applications (50 to 125 kW), because of their fast warm-up/start-up time, low sensitivity to orientation, and favorable power-to-weight ratio (Figure 1.3). However they can be found also in some stationary applications, from homes (1-5 kW) to electrical generation plants (up to 250 MW or more).

The system cost is one of the disadvantages of the PEMFC, due mainly to the noble-metal used as catalyst (platinum). Secondly, they show sensitivity to impurities in the fuel source, especially carbon monoxide (CO): even tens of parts per million of CO can poison a pure platinum catalyst, therefore the addition of a pre-reactor to reduce CO in the fuel gas is necessary (if H_2 is derived from an alcohol or hydrocarbon fuel), together with the development of more resistant alternatives, such as platinum/ruthenium combination. Another obstacle, specific for their use in vehicles, is the pure-hydrogen storage on-board in pressurized tanks. Due to the low-energy density of hydrogen, it is difficult to store enough hydrogen on-board to allow vehicles to travel the same distance as gasoline-powered vehicles before refueling, typically 300-400 miles. A solution could be the use of an on-board reformer, which convert higher-density liquid fuels (such as methanol, ethanol, natural gas, liquefied petroleum gas, and gasoline) to hydrogen. Nevertheless this would increase costs and maintenance and release carbon dioxide.

Fig. 1.3 A fuel cell car powered by hydrogen and sold by Hyundai®.

- High-temperature fuel cells

Among the fuel cells working at high temperature, the solid oxide fuel cells (SOFCs) (Figure 1.2) are a particularly promising

technology. Thanks to higher electric efficiency and high temperature of co-generated heat, to the possibility of realizing generators of good size (up to some MWe), to the capability of operating with different fuels (hydrogen, methane, bio-fuels, etc.) and to high tolerance to gas impurities, SOFCs are better than other FCs (both LT and HT) to adapt to stationary co-generation applications.

SOFCs use a hard, non-porous ceramic compound as electrolyte and differently from PEMFC the electrolyte conduct negative oxygen ions from the cathode to the anode, where they can be electrochemically oxidized with hydrogen or other fuels. The solid nature of the electrolyte allows SOFC to be constructed in different configurations than the typical plate shape of other FCs (Figure 1.4).

SOFCs are expected to be around 50%-60% efficient at converting fuel to electricity and with the exploitation of the co-generated waste heat they could reach overall efficiencies of 80%-85%.

Solid oxide fuel cells operate at very high temperatures, around 1,000 °C, which allows to remove the need of precious-metal catalyst, thereby reducing cost. It also allows SOFCs to reform fuels internally, thus enabling the use of a variety of fuels and reducing the cost associated with addition of a reformer to the system. However, high-temperature operation has also disadvantages: it results in a slow start-up and requires significant thermal shielding to retain heat and protect personnel, which may be acceptable for utility applications but not for transportation and small portable applications (U.S. D.o.E., 2001). The high operating temperatures also deeply affects the durability requirements on materials, and so low-cost materials with good durability at high operating temperatures are needed. Simultaneously, researchers are studying an alternative strategy: SOFCs operating at lower temperatures would have fewer durability problems and lower costs for materials, even if they would probably produce less electrical power.

Another big advantage of the SOFCs is their resistance-to-contaminants: they can tolerate several orders of magnitude more sulfur than other cell types and, in addition, they are not reported to be poisoned by carbon monoxide (CO), which can even be used as fuel. This property makes SOFCs the preferred FC technology to use gases made from coal or renewable sources like biomasses.

Fig. 1.4 Bloom Boxes® by Bloom Energy Corporation of Sunnyvale, California, USA. On-site "energy servers" using SOFCs for distributed power, each one producing 100 kW of electricity.

1.3 Bio-Hydrogen

1.3.1 Biological processes for hydrogen production

Biological production of hydrogen is known since the late 1800, when basic research established that algae and bacteria could produce hydrogen (Jackson and Ellms, 1896). With the oil crisis of the early '70s, the National Science Foundation (NSF, Washington, DC, USA) sponsored several meetings on biological hydrogen production, which firstly roused the interest about the matter, especially focusing on photosynthetic processes (Benemann, 1996). With the renewal of interest in the '90s about renewable energy sources, biological hydrogen production became a focus of

additional governmental support, particularly in Germany, the US and Japan, with minor efforts in other countries. This led to an enlargement in the biohydrogen production scenario, including also strategies different than photosynthetic/photolytic processes (Kotay and Das, 2008).

In general biological hydrogen production processes have some advantages over their chemical or electrochemical counterparts. They are catalyzed by microorganisms in an aqueous environment at ambient temperature and pressure and are well suited for decentralized energy production in small-scale installations in locations where biomass or wastes are available, thus avoiding energy expenditure and costs for transport.

From a thermodynamic perspective, as the organic substrates dissolved and diluted in wastewater are in a high entropy state, it is somewhat difficult to obtain their combustion enthalpy by mechanical means (Kotay and Das, 2008). On the contrary microorganisms can naturally recover and concentrate the energy from high water content organic resources, such as industrial wastewater and sludge in a usable form. Thus, biohydrogen production is an entropy reducing process, that could not be realized by mechanical or chemical systems (Nandi and Sengupta, 1998).

Broadly speaking, the variety of biological processes for hydrogen production can be split in light-dependent or light-independent processes. Light mediated processes include direct or indirect biophotolysis and photosynthetic strategies (photofermentation and microbial water shift), whereas dark fermentation is the major light independent process. Figure 1.5 describes from a biochemical point of view these different bio-hydrogen production approaches and additional information about each of them will follow.

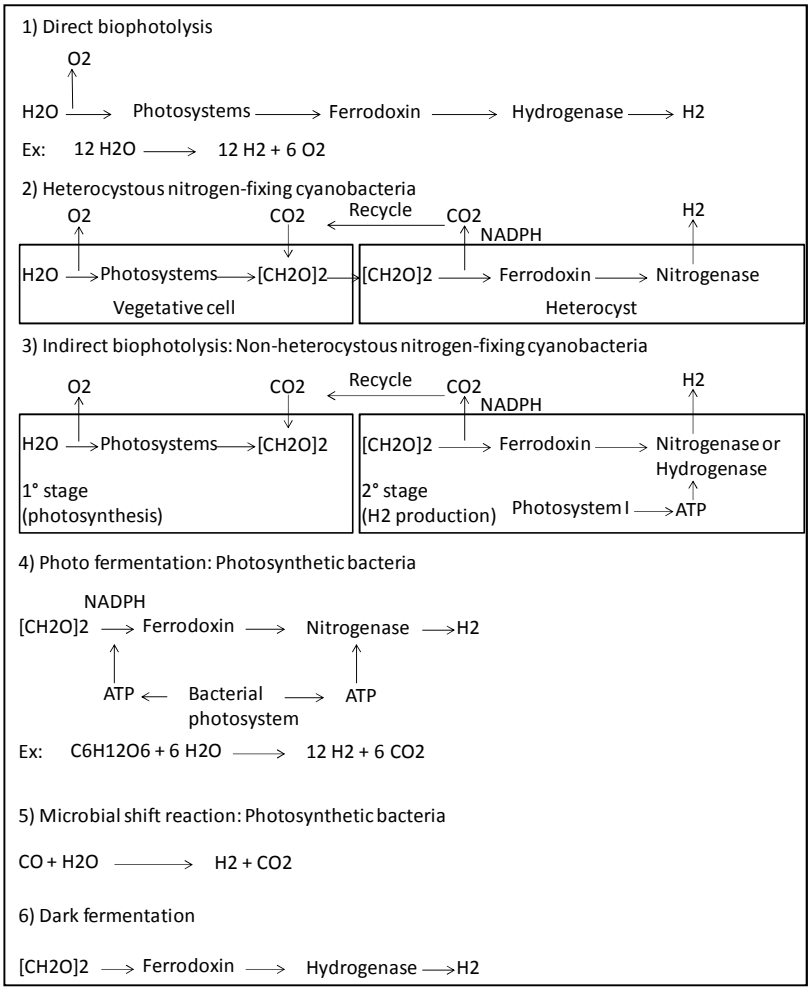

Fig. 1.5 Biological hydrogen production approaches.

It must be said that it is too early to predict which of them will be ultimately successful, or how they would appear in case of large-scale production processes or small scale conversion devices, because their practical development still requires scientific and

technological advances, and middle- to long-term applied R&D (Benemann, 1996).

For example, the group of bio-technologies based on photosynthetic systems refers to a theoretically perfect process, directly transforming solar energy into hydrogen by photosynthetic bacteria. For real, due to the low utilization efficiency of light and difficulties in designing light reactor, this strategy is hard to be applied in practice (Liu *et al.*, 2008). Indeed, the reducing power generated by photosynthesis must be produced as close as possible to the maximal possible solar conversion efficiencies (about 10%) and then efficiently transferred to hydrogenase enzyme. On the contrary, photosynthetic organisms like higher plants currently capture only 3-4% of sunlight's available energy at most (Benemann, 1996).

1 - Direct biophotolysis

The (direct) splitting of water to generate hydrogen by solar radiation is a process achieved either in photochemical cells where, for example, TiO_2 is illuminated as the catalyst, or by applying photovoltaics, which indirectly utilize solar radiation for the electrolysis of water into H_2 and O_2 (Kotay and Das, 2008).

A biological alternative to this process is the direct biophotolysis, which involves light-driven decomposition of water through micro-algae or cyanobacteria (Benemann, 1996). Green algae are able to evolve hydrogen by means of a reversible hydrogenase which receives the reductants generated by photosynthesis from a reduced ferrodoxin. On one hand, under laboratory condition at low light intensities, it has been demonstrated that the green alga *Chlamydomonas* converts up to 22% of light energy into hydrogen energy, equivalent to a 10% solar energy conversion efficiency (Greenbaum, 1988). On the other hand, this process is based on two low compatible reactions: in the first step, water is split to produce oxygen, and in the second, the reducing power of electrons is passed to protons to make hydrogen through hydrogenase. Since oxygen is a

strong inhibitor of hydrogenase activity, a feedback inhibition mechanism is inherent in the system. Aiming at real world application, this is a great obstacle: since laboratory strategies to overcome it by either consuming or sweeping out the oxygen as fast as it is produced would not be practical for large scale operations, the only applicable strategy could be the cultivation of algae under sulfur deprivation for 2 or 3 days to provide anaerobic conditions in the light (Winkler *et al.*, 2002).

2 - Heterocystous nitrogen-fixing cyanobacteria

This strategy assumes to employ algae (such as *Anabaena cylindrica*, a filamentous cyanobacteria) as systems where the two previously described incompatible reactions can be separated by compartmentalization, with CO_2 acting as an intermediate to shuttle between the two compartments. In this type of organisms, oxygenic photosynthesis is restricted to vegetative cells, then microoxic heterocysts evolves hydrogen from reductants generated by photosynthesis through the use of ferrodoxin and nitrogenase enzymes, after N_2 reduction process has been blocked. Nitrogenase is the key enzyme that catalyzes hydrogen gas production and it must be noticed that its activity is inhibited in the presence of oxygen, ammonia or at high N/C ratios (Kapdan and Kargi, 2006)

3 - Indirect biophotolysis: non-heterocystous nitrogen-fixing cyanobacteria

Differently from the previous strategy developed by heterocystous cyanobacteria, non-heterocystous nitrogen-fixing cyanobacteria are able to separate the H_2 and O_2 evolution steps temporally, such as a day-night cycle, or spatially, through separate bioreactions rather than through two cell types. However, as before, CO_2, which is fixated and released, is the intermediate between the two reactions.

Moreover, these microorganisms can use both nitrogenase and reversible hydrogenase for hydrogen production: unfortunately

nitrogenase has a high ATP requirement, which lowers potential solar-energy conversion efficiencies to unacceptable levels. Considering the real-world applicability of this process (which has been already tested in a two-stage power plant in Osaka, Japan, by Akano *et al.*, 1996), the advantage is that the CO_2 fixation stage, representing up to 90% of the total area required for the plant, would take place in open ponds, which are much cheaper than the closed photobioreactors typically required for the H_2 photo-evolution stage.

4 - Photo fermentation: photosynthetic bacteria

In the light, some photosynthetic bacteria can convert organic substrates, including many wastes, into hydrogen and CO_2 (Figure 1.5, pathway 4). In principle relatively little light-energy input (which means small photobioreactors) should be required to drive this reaction, as most of the hydrogen energy comes from the organic substrates themselves. However, the high-energy demands by the nitrogenase catalyzing hydrogen evolution in these bacteria and the relatively low light intensities at which these bacteria operate, make their photosynthetic efficiencies disappointing.

Hydrogen production rates vary depending on the light intensity, carbon source and the type of microbial culture. The organisms seem to prefer organic acids as carbon source, such as acetic, butyric, propionic, lactic and malic acid. On the basis of available literature, the highest conversion efficiency, between 80 and 86%, was obtained using lactic acid as the carbon source (Kapdan and Kargi, 2006). Carbohydrates and industrial effluents may also be used: using three different substrates hydrogen was produced by four strains of photofermentative *Rhodopseudomonas sp.* bacteria. Among them, sugarcane juice supported the maximum level of hydrogen production followed by potato starch and whey at the rates of 45, 30 and 25 ml H_2 $mg^{-1}_{\text{bacterial cell(dry weight)}}$ h^{-1}, respectively (Ike *et al.*, 1999).

5 - Microbial shift reaction: photosynthetic bacteria

Photosynthetic bacteria can also act as biological catalysts in the conversion of carbon monoxide to hydrogen, a strategy which has the potential for near term practical application. The microbial shift reaction shown in Figure 1.5 (pathway 5) can accomplish this conversion at room temperature and in just one step (in contrast to chemical catalysts use). This pathway could be particularly useful for small scale application, but in order to make this process economically feasible it would be advantageous to use gas phase bioreactors and overcome the mass transfer limitation (Markov *et al.*, 1996).

6 - Dark fermentation

Dark fermentative hydrogen production has a lower technical complexity compared to the photo fermentation process and can exploit a broad spectrum of applicable substrates with high hydrogen evolution rate (Nath and Das, 2004). The word "dark" is used to distinguish this technology from other fermentations that need light to work. This technology will be discussed in details in the following section (1.3.2).

Tab. 1.1 Biological hydrogen production processes.

Process	Advantages	Disadvantages	Type of organism - *Representative strain*	Maximum reported rate (mmol H_2/L h)	References
Direct Biophotolysis	Can produce H_2 directly from water and sunlight	Requires high intensity of light	Green algae - *Chlamydomonas Reinhardii*	0.07	Levin et al. 2004; Mahyudin et al. 1997
	Solar conversion energy increased by tenfolds as compared to trees and crops	O_2 can be dangerous for the system			
		Lower photochemical efficiency			
Indirect Biophotolysis	Can produce H_2 from water	Uptake hydrogenase enzymes are to be removed to stop degradation of H_2	Cyanobacteria - *Anabaena Variabilis*	0.36	Tanisho et al. 1998; Kataoka et al. 1997
	Has the ability to fix N_2 from atmosphere	About 30% O_2 present in gas mixture			
		O_2 has an inhibitory effect on nitrogenase			
Photofermentation	A wide spectral light energy can be used by these bacteria	Light conversion efficiency is very low, only 1-5%	Photosynthetic bacteria - *Rhodobacter Spheroides*	0.16	Mizuno et al. 2000
	Can use different waste materials like distillery effluents, waste etc	O_2 is a strong inhibitor of hydrogenase			
Dark fermentation	It can produce H_2 all day long without light	Relatively lower achievable yields of H_2	Fermentative bacteria - *Enterobacter cloacae DM 11*	75.6	Hussy et al. 2003
	A variety of carbon sources can be used as substrates	As yields increase H_2 fermentation becomes thermodynamically unfavorable	*Clostridium sp. Strain* No 2	64.5	Lay 2000
	It produces valuable metabolites such as butyric, lactic and acetic acids as by products	Product gas mixture contains CO_2 which has to be separated			
	It is anaerobic process, so there is no O_2 limitation problem				

1.3.2 Hydrogen production by Dark Fermentation

Dark fermentative hydrogen (Pathway 6, Figure 1.5) is produced by the intermediate steps (acidogenesis and acetogenesis) of the well known Anaerobic Digestion (AD) process, and therefore it shares some of the advantages of this well-established technology, like the capability of exploiting low-cost substrates/organic wastes at high yields.

Before delving into the hydrogen production, it is essential to briefly present the AD process, a complex microbiological process involving a community of several populations and many different metabolic pathways.

As known, microbial conversions of organic matter occur in sequence from more to less energetically favorable reactions and if several electron acceptors (oxygen, nitrate, sulfate, iron (III), and CO_2) are available, the most energetically favorable electron acceptor will be utilized. So in the absence of the electron couple O_2/H_2O (aerobic respiration, $\Delta G_H^0 n^{-1}$ = -78.3 kJ/mol) or other strong acceptors such as in AD, the organic matter will be reduced to the end products CH_4 and CO_2 via methanogenesis ($\Delta G_H^0 n^{-1}$ = +23.5 kJ/mol).

The AD could be divided into four main steps, as follows:

- *Hydrolysis*: conversion of non-soluble biopolymers to soluble organic compounds;
- *Acidogenesis*: conversion of soluble organic compounds to volatile fatty acids (VFA) and CO_2;
- *Acetogenesis*: conversion of volatile fatty acids to acetate and H_2;
- *Methanogenesis*: conversion of acetate and H_2 to methane gas.

Figure 1.6 gives a schematic representation of anaerobic degradation of organic matter.

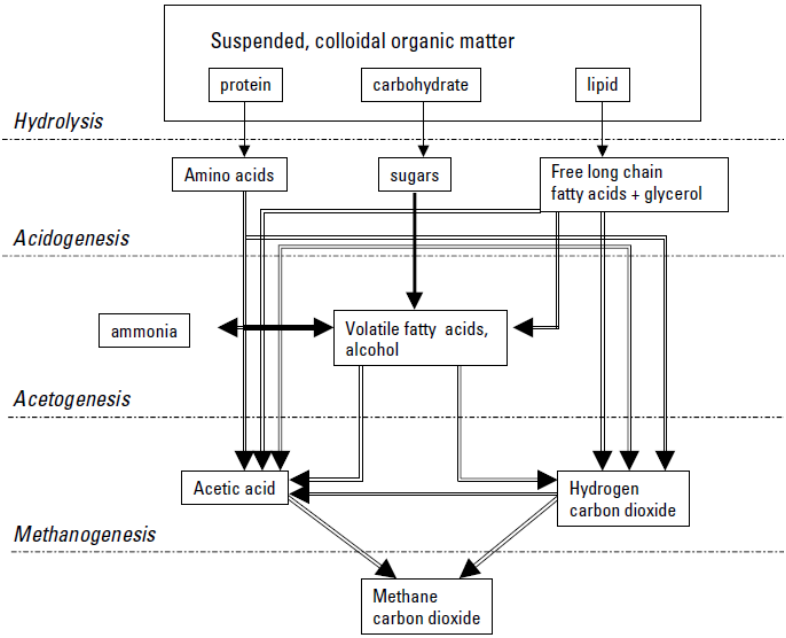

Fig. 1.6 Simplified schematic representation of the anaerobic digestion process.

A great advantage of fermentation is the fast degradation of solids and other complex organics, such as those found in wastes and agricultural products. But despite its speed, fermentation is not yet very efficient for capturing the energy value of biomass into hydrogen (Kotay and Das, 2008). Indeed, the main problem is that the dark fermentative bacteria produce only relatively small amounts of hydrogen and, as yield increase, hydrogen fermentation becomes thermodynamically unfavorable, decreasing the feasibility of its application to an industrial scale. Benemann (1996) estimated that fermentative hydrogen production from wastewater has the greatest potential for economical near-term production of bio-hydrogen, but only if hydrogen conversion efficiency could reach 60-80%.

If biomass could be categorized as a carbohydrate such as glucose ($C_6H_{12}O_6$), the complete conversion of each mole of glucose would produce 12 mol of hydrogen (Equation 1).

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

The maximum result about fermentative hydrogen yield was reported by Woodward *et al.* (2000) who achieved a 96.7% conversion efficiency (11.6 $mol_{H2}/mol_{glucose\text{-}6\text{-}phosphate}$) by combining the enzymes of the oxidative pentose phosphate cycle with hydrogenases from *Pyrococcus furiosus* under optimized in vitro conditions. However this result was obtained by using exclusively enzymes, not bacteria. Indeed, in bacterial fermentation the complete oxidation of glucose into hydrogen and carbon dioxide is not possible, as the corresponding reaction (1) is not feasible thermodynamically, having a positive ΔG_o of +3.2 kJ. According to Logan (2004) fermentation of glucose by all known microbiological routes (primarily by *Clostridia*) produces up to 4 $mol_{H2}/mol_{glucose}$. Thus, there are no known naturally occurring biochemical routes for achieving the required 60-80% conversion efficiency.

Moreover, while a conversion efficiency of 33% is theoretically possible for laboratory hydrogen production from glucose (corresponding to 4 mol H_2/mol glucose), only half of this is usually obtained under batch or continuous fermentation conditions (Logan *et al.*, 2002; Van Ginkel *et al.*, 2001). Indeed, glucose gives 4 mol of hydrogen if exclusively 2 mol of acetate are simultaneously produced (Equation 2), while only 2 mol H_2 are achieved when butyrate is the main fermentation product (Equation 3).

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 4\ H_2 + 2\ CO_2 + 2\ C_2H_4O_2 \quad (2)$$

$$C_6H_{12}O_6 \rightarrow 2\ H_2 + 2\ CO_2 + C_4H_8O_2 \quad (3)$$

In particular, according to Liu *et al.* (2002), using mixed microbial cultures for inoculation of the process, a combined production of both acetic and butyric acid often occurs, with butyrate typically amounting to 60-70% of the aqueous products (Equation 4):

$$4\ C_6H_{12}O_6 + 2\ H_2O \rightarrow 8\ H_2 + 8\ CO_2 + 3\ C_4H_8O_2 + 2\ C_2H_4O_2 \quad (4)$$

This results again in a maximum hydrogen conversion yield of 2 $mol_{H2}/mol_{glucose}$.

Moreover, other compounds may be present as end products of the fermentation, further lowering the yields. If propionic acid is the end product, the H_2 conversion yield is just 1 $mol_{H2}/mol_{glucose}$:

$$C_6H_{12}O_6 \rightarrow H_2 + CO_2 + C_3H_6O_2 \quad (5)$$

while the formation of lactic acid ($CH_3CHOHCOOH$) by anaerobic degradation of glucose is associated with no production of hydrogen (Equation 6).

$$C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3 \quad (6)$$

In addition lactic acid bacteria (e.g. *Lactobacillus paracasei* or *Enterococcus durans*) can form intermediate catabolic products (so-called bacteriocrine), inhibiting hydrogen producing bacteria (Noike *et al.*, 2002).

Another reason suggested for lower yields is that glucose is usually just partially biodegraded through this bioprocess (Kapdan and Kargi, 2006). However, it is more probable that utilization of substrate for bacterial growth is the main reason for obtaining yields lower than theoretical estimations, because it was shown that even when more than 95% glucose was degraded, the yield could be less than 1.7 mol H_2/mol glucose (Lin and Chang, 2004).

In Paper II we collected the best results in terms of yield ($mol_{H2}/mol_{glucose}$) reported in the literature for glucose dark fermentation in batch test with mixed microbial cultures or with pure/selected/GM microorganisms (Figure 1.6; see also Table S.2 in supplementary information section of Paper II). H_2 yield obtained in our research (Paper II) by mixed microbial cultures enriched from natural sources (soil-inocula and anaerobically digested materials) are reported in the same figure. With low substrate concentration (1 g

$_{glucose}$/L) we achieved high H_2 yields (2.8 ± 0.66 $mol_{H2}/mol_{glucose}$), comparable to pure microbial cultures achievements.

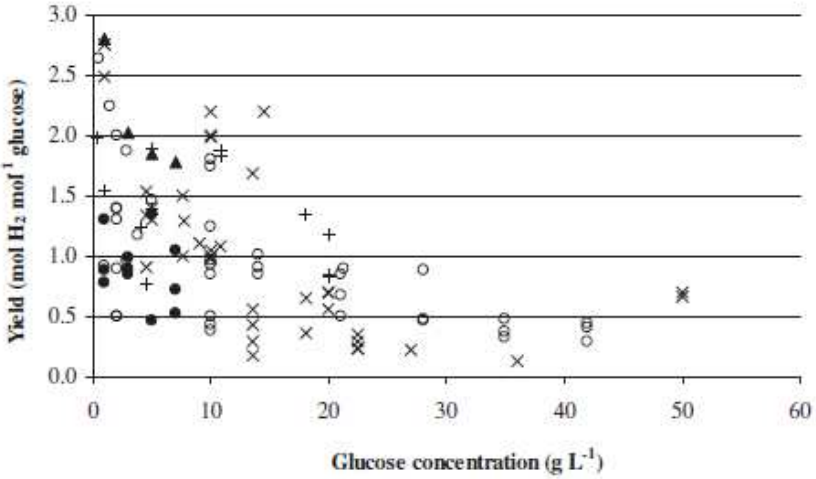

Fig. 1.6 Effect of substrate concentrations and type of inoculum on hydrogen yield: our Paper II results in comparison with literature results. (▲) acclimated inocula (Paper II), (●) soil inocula (Paper II), (○) literature results with naturally-sourced mixed microbial cultures, (×) literature results with pure/selected wild type microbial cultures and (+) literature results with genetically modified pure cultures.

Tab. 1.2 Reported maximum fermentative hydrogen yield achieved by dark fermentation in continuous system (with or without optimization efforts). Full references are reported in the Reference section of this Ph.D. thesis.

Process	Substrate	Max H_2 yield (mol H_2/mol hexose)	References
Fluidized bed reactor	Sucrose	1.3	Wu et al. 2003
N_2 sparging, CSTR	Wheat starch co-product	1.9	Hussy et al. 2003
Upflow reactor	Wastewater	2.1	Yu et al. 2002
Fermentor	Sucrose	2.1	Fang et al. 2002
Fermentor	Glucose	2.1	Fang and Liu 2002
CSTR	Glucose, sucrose	2.2	Chen and Lin 2001
N_2 sparging, CSTR, HRT 8.5 h	Glucose	1.43	Mizuno et al. 2000
N_2 sparging, continuous	Glucose	1.4-2.3	Kataoka et al. 1997
Chemostate, HRT 17 h	0.75% soluble starch	2.14	Lay 2000
Continuous (immobilized bioreactor)	Glucose	2.3	Kumar and Das 2000
Continuous (immobilized bioreactor)	Glucose	3.8	Kumar et al. 2001

Similarly, Table 1.2 collects the recent maximum hydrogen yield achieved in continuous experiments, also including optimization efforts. Again, those yields typically reach 2.1 mol_{H2}/mol_{hexose}.

However, Benemann (1996) stated also that the economics of hydrogen fermentation could be favorable even at less then stoichiometric yields: production costs of methane fermentations range from $3 to $8 per MMBTU (million British thermal units), whereas hydrogen produced by the same types of hardware could be sold as much as $15 per MMTBU, depending on location, scale, purity and other factors. For real, even if hydrogen fermentation would embrace similar hardware to that currently used in industrial anaerobic digesters, economic feasibility will not be sustainable until these yields reach the 60-80% efficiency mark, even without taking into account the substrate used for the process. It has been speculated that a fermentation yield of 10 $mol_{H2}/mol_{glucose}$ and a glucose cost of 5 cents per dry pound will be required for this process to approach costs competitive with traditional fuels.

Based on these considerations, it is possible to summarize and to split in three different strategies the possible ways to increase the biological energy recovery via hydrogen dark fermentation:

1. Increasing the biohydrogen yield to around 85% through chemical-physical process conditions optimization, efficient bioreactor design, use of suitable microbial strains, genetic and metabolic engineering of hydrogen-producing microorganisms, redirection of the metabolic pathways, etc. (details in section 1.3)
2. Using essentially cost-free substrates like solid waste materials, such as those from farms, and dissolved organic matter from various industrial and domestic wastewaters (details in chapter 2)
3. Finding methods to harness the remaining 85% of the energy, i.e. including hydrogen production into a modular energy production concept (details in chapter 4).

1.4 Key parameters regulating the dark fermentative hydrogen production and their optimization

Process engineering, such as bioreactor design and operating parameters optimization, may enhance the performances of the fermentative hydrogen-producing microorganisms, thus positively affecting the final hydrogen yield. Also, the unstable hydrogen production could be minimized, since it is possibly attributed to the metabolic shift of bacteria or system imbalance.

Some regulating factors among those that can be manipulated to steer a bioconversion process to the desirable product (in this case, hydrogen) would be discussed in this section.

- *Substrates (pure)*

Considering their higher yields of hydrogen per mole of substrate, carbohydrates are the preferred pure substrate for laboratory-scale dark fermentative hydrogen production. They can be monosaccharides, but may also be polymers such as starch, cellulose or xylan.

Table 1.3 (Courtesy of Kapdan and Kargi, 2006) summarizes the yields and the rates of hydrogen production obtained from batch and continuous tests when hexoses (glucose and sucrose) and polysaccharides (starch and cellulose) were used as the substrate.

Dealing with carbohydrates polymers and according to the reaction stoichiometry, a maximum of 553 mL of hydrogen gas can be produced from 1 gram of starch, with acetate as a by-product. Considering that the yield may be lower than the theoretical value because of utilization of substrate for cell synthesis, Zhang *et al.* (2003) obtained a noticeable result, with high specific yield of 480 mL_{H2}/g_{VSS} d feeding a mixed sludge with 4.6 g_{starch}/L solution. Very high hydrogen yield (2.4 $mol_{H2}/mol_{glucose}$) was also obtained by

mixed culture from starch in the presence of 0.1% polypepton (Yokoi *et al.*, 2001).

Tab. 1.3 Yields and rates of bio-hydrogen production from pure carbohydrates by batch or continuous dark fermentations (Courtesy of Kapdan and Kargi, 2006).

Batch experiments

Organism	Carbon source	SHPR	VHPR	H_2 yield	H_2 content in gas mixture (%)
Klebsielle oxytoca HP1	Glucose (50 mM)	9.6 mmol/g DW h	87.5 mL/L h	1 mol/mol glucose	
E. cloacae IIT-BT 08	Glucose (1%)		447 mL/L h	2.2 mol/mol glucose	
E. coli	Glucose (20 g/L)			4.73×10^{-8} mol/mol glucose	
H. alvei	Glucose (10 g/L)			5.87×10^{-8} mol/mol glucose	
Sludge compost	Glucose (10 g/L)		147 mL/L h	2.1 mol/mol glucose	
Mixed culture	Glucose (1 g COD/L)			0.9 mol/mol glucose	60
Mixed culture	Sucrose (6 g/L)	9 mL/g VSS h		300 mL/g COD	40
Klebsielle oxytoca HP1	Sucrose (50 mM)	8.0 mmol/g DW h		1.5 mol/mol sucrose	
C. pasteurium (dominant)	Sucrose (20 g COD/L)	4.58 mmol/g VSS h	270 mmol/L d	4.8 mol/mol sucrose	55
E. cloacae IIT-BT 08	Sucrose (10 g/L)	29.5 mmol/g DW h	660 mL/L h	6 mol/mol sucrose	92
Mixed culture	Sucrose (1 g COD/L)			1.8 mol/mol sucrose	
Thermoanaerobacterium	Cellulose (5 g/L)	11.9 mL/g VSS h		102 mL/g cellulose	
Clostridium sp.	Microcristalline cellulose (25 g/L)	0.46 mmol/VSS d		2.18 mmol/g cellulose	60
E. aerogenes	Starch[a] (20 g glucose/L)	9.68 mmol/g DW h	17.4 mmol/L h	1.09 mol/mol glucose	
Thermoanaerobacterium	Starch (4.6 g/L)	15.2 mL/g VSS h	1.9 mL/h	92 mL/g strach	60
C. pasteurium	Starch (24 g/L)	9.9 mL/gVSS h	4.2 mL/h	106 mL/g starch	
Mixed culture	Potato starch (1 g COD/L)			0.59 mol/mol starch	
Mixed culture	Sugar beet juice			1.7 mol H_2/mol hexose	

Continuous experiments

Organism	Carbon	SHPR	VHPR	H_2 yield	% H_2 content	Reactor	HRT_f (h)
C. acetobutyricum	Glucose	6 mmol/OD_{600} h L		2 mol/mol glucose	50	Fed-batch	
Mixed culture	Glucose (20 g COD/L)	20 mmol/g VSS h		1.1 mol/mol glucose		CSTR	4
Mixed culture	Glucose (13.7 g/L)		376 mmol/L d	1.2 mol/mol glucose	60	Trickling biofilter	4–12
Clostridia sp.	Glucose (20 g COD/L)	14.2 mmol/g VSS h	359 mmol/L d	1.7 mol/mol glucose	42.6	CSTR	6
Mixed culture	Glucose (7 g/L)	191 mL/g VSS h		2.1 mol/mol glucose	64	CSTR	6
Mixed culture	Glucose (20 g/L)		300 mL/L h		60	UASB	20
Clostridium sp.	Glucose (10 g/L)		640 mL/h		60	AMBR[a]	3.3
E. aerogenes HO39	Glucose (10 g/L)		850 mL/L h			Fixed film	1
Mixed culture	Sucrose (20 g COD/L)		105 mol/h	3.47 mol/mol sucrose		CSTR	8
Mixed culture	Sucrose	340 mL/g VSS h	5.10 L/h L	2.1 mol/mol sucrose	35	CIGSBR[b]	0.5
Mixed culture	Sucrose (20 g COD/L)	2.2 mmol/g VSS h	270 mmol/L d	1.5 mol/mol sucrose	42	UASB	8
Mixed culture	Sucrose (20 g COD/L)	3.7 mmol/gVSS h	470 mmol/L d	2.6 mol/mol glucose	35	SBR	4–12
Klebsiella oxytoca HP1	Sucrose (50 mM)	15.2 mmol/g DW h	350 mL/L h	3.6 mol/mol sucrose		CSTR	5
Mixed culture	Sucrose (20g COD/L)	35 mmol/g VSS h	20.8 L/L d	1.48 mol/mol sucrose	42	CSTR	2
C. butyricum + *E. aerogenes*	Starch (2%)	NA	800 mL/L h	2.5 mol/mol glucose		CSTR	2
C. butyricum + *E. aerogenes*	Starch (2%)	NA	1300 mL/L h	2.6 mol/mol glucose		Immobilized[c]	0.75
Thermococcus kodakaraensis KOD1	Starch (5 g/L)	14.0 mmol/g DW h	9.46 mmol/L h	3.33 mol/mol starch	<10	Gas-lift fermenter	5
Mixed culture	Wheat starch (10 g/L)		131 mL/L h	0.83 mol/mol starch d	50.3	CSTR	12
Mixed culture	Starch (6 kg starch/m^3)	97.5 mL/g VSS h	1497 L/m^3 d	1.29 L/g starch COD	61	CSTR	20
C. termolacticum	Lactose (29 mmol/L)	5.74 mmol/g DW h	2.58 mmol/L h	3 mol/mol lactose	86	CSTR	5–35

[a] Anaerobic membrane bioreactor.
[b] CIGBR, carrier induced granular bed reactor.
[c] Immobilization on porous glass beads; SHPR, specific hydrogen production rate; VHPR, volumetric hydrogen production rate.

On the other side, proteins, peptides and amino acids seem to be less suitable for dark hydrogen production, whereas biopolymers like lipids are unsuited. Formate and some peptides have been studied before as substrates for dark hydrogen production by De Vrije and

Claassen (2005), who obtained amino acids oxidation to hydrogen by specific strains of extreme thermophilic bacteria. However it is still unclear whether specific amino acids, entering bacterial metabolism at the level of pyruvate, are selected or whether this phenomenon is more general.

The vast range of potential organic substrates for dark hydrogen fermentation will be dealt also in chapter 2 of this Ph.D. thesis, specifically referring to complex substrates such as agricultural biomasses and actual organic waste streams (from solid wastes to wastewaters) which have been successfully used for H_2 production.

- *Biomass pre-treatment processes*

Hydrolysis is the first step of the biologic conversion of biomass. During the hydrolysis, both solubilization of insoluble particulate matter and biologic decomposition of organic polymers to dimers and monomers (e.g. simple sugars, amino acids, long-chain fatty acids and aromatic compounds) take place. This step is especially required for recalcitrant biomasses like the lignocellulosic ones and it may be accomplished biologically or by means of chemical and/or physical techniques. So, a number of pre-treatments can be chosen, depending on the biomass solid structure, the process used for biomass fermentation and the desired microbial products. It is possible to include (Gavala *et al.,* 2003):

- Heat treatment: in the temperature range of 40-275 °C. In case of lignocellulosic biomasses, among celluloses, hemicelluloses and lignin, the hemicelluloses are the most sensitive to the thermal treatment and thus are the first to be degraded (Ntaikou *et al.*, 2010a), with just a partial solubilization of lignin at 160 °C or above.
- Chemical treatment: using ozone, acids, alkali, etc. Dealing with lignocellulosic biomass, both dilute or concentrated acids, such as H_2SO_4 and HCl, can be used. Mainly hemicellulose is hydrolyzed in this process, while lignin is hardly dissolved,

nevertheless being disrupted to a high degree it leads to increased cellulose susceptibility to enzymes. In case of alkaline pretreatment, diluted bases cause the decrease of polymerization degree and crystallinity, the destruction of links between lignin and other polymers, and the lignin breakdown.

- Mechanical treatment: using ultrasounds, mills or homogenizers. Mechanical pre-treatment is almost always applied before any other kind of pre-treatment, and aims at the reduction of particle size and crystallinity of biomass, leading to an increased specific surface available for enzymatic attack.
- Biological hydrolysis (enzyme addition): specific enzymes may drastically improve the depolymerization of the organic matter. Indeed, either enzymes -hydrolases - or extracellular enzyme-producing microbial cells may be added to accomplish biomass hydrolysis, as typically occurring in the food processing industry, where enzymes are used in order to decrease food wastes with simultaneous formation of higher value chemicals or biofuels (in particular, production of ethanol from agricultural and forestry residues) (Galbe and Zacchi, 2002). Biological pre-treatment seem very effective especially with lignocellulosic biomass, proceeding through the concerted action of specific enzymes, i.e. lignin peroxidase, manganese peroxidase, H_2O_2-generating enzymes and laccase, which produce strong oxidants and combust the lignin framework.
- Combination of two or more of the aforementioned methods: for example wet oxidation (combination of oxygen pressure, alkaline conditions and elevated temperature) of the lignocellulosic material has been used to enhance the accessibility of the carbohydrates, as it dissolves the hemicellulose fraction and makes the solid cellulose fraction susceptible for enzymatic hydrolysis and fermentation (Lyberatos *et al.*, 2005).

- *Fermentation pathways and their terminal products*

The dark fermentation of organic wastewaters is basically of three types: butyric acid-type fermentation, metacetonic acid-type fermentation, and ethanol-type fermentation. Each fermentation has specific properties and terminal products that can affect hydrogen-producing ability and metabolic pathways of fermentative microflora. For example, ethanol fermentation achieves high ethanol production with simultaneously high hydrogen production (specifically: higher biogas and H_2 production rate and higher hydrogen content than metacetonic acid-type fermentation) under equal quantities of aqueous terminal products. As showed by Ren *et al.* (2006) ethanol seems to have little inhibitory effect on fermentative hydrogen production, while acetic acid has strong inhibitory effect on hydrogen production.

However, the common major products in hydrogen production by anaerobic dark fermentation of carbohydrates are acetic, butyric and propionic acids, and even formation of lactic acid was observed when lactose and molasses (sucrose) were used as substrates (Kapdan and Kargi, 2006). High concentration of organic acids may result in a collapse of the pH gradient across the membrane and cause the total inhibition of all the metabolic functions in the cell (Jones and Woods, 1986).

It has been claimed that both the total acetate or butyrate acid concentration and the undissociated form of these acids can inhibit the dark hydrogen fermentation process (Jones and Woods, 1986; Van Ginkel and Logan, 2005b; Van Niel *et al.*, 2002). In particular, Van Ginkel and Logan (2005b) studied the inhibition of biohydrogen production by using undissociated acetic and butyric acids: with acetic acid addition to give total undissociated acid concentrations in the reactor of 63 mM, which occurred at pH 5.5 and 165 mM of added acetate, complete H_2 production inhibition was reached. In Liu *et al.* (2008) hydrogen yields were inhibited more by self-produced

acids than by similar concentrations of externally added acids and the acetate concentration started to inhibit extreme-thermophiles hydrogen production at more than 50mM. At acetate concentration of 200 mM, the hydrogen production (36 mL/$g_{VSadded}$) was 7 time lower than at 5-25 mM acetate (250 mL/$g_{VSadded}$), and the lag phase was more than 100 hours.

- *Microorganisms*

Hydrogen production can be achieved either through selected hydrogen producing bacteria (pure cultures of strict anaerobes, facultative anaerobes and even some aerobes) or mixed microbial cultures derived from natural environments (soil, wastewater sludge, compost...) (Liang, 2002, Nandi and Sengupta, 1998).

The advantages of pure cultures are the selectivity of substrates, the easy manipulation of the metabolism by altering growth conditions, the common high hydrogen yields as an effect of the reduction of undesired by-products and the repeatability of the process. On the other hand, they are sensitive to contaminations, thus requiring aseptic process conditions unfeasible for industrial production of H_2 (Ntaikou *et al.*, 2010a).

Among the obligate anaerobes spore-forming organisms (Clostridia, methylotrophs, rumen bacteria, archaea), those belonging to the genus *Clostridium* (*C. buytricum*, *C. thermolacticum*, *C. pasteurianum*, *C. paraputrificum* M-21 and *C. bifermentants*) are widely exploited. They produce hydrogen gas during the exponential growth phase but when the stationary growth phase is reached, their metabolism could shift from a hydrogen/acid production phase to an undesirable solvent production phase (Kapdan and Kargi, 2006). Interestingly, a culture with *Clostridia* dominance can be easily obtained by heat treatment of biological sludge: the spores formed at high temperatures can be activated when required environmental conditions are provided for hydrogen gas production (Sung *et al.*, 2003).

Among the facultative anaerobes, the species of the genus *Enterobacteriaceae* have the ability to metabolize glucose by mixed acid or the 2-3 butanediol fermentation pathway. In both patterns, CO_2 and H_2 are produced from formic acid in addition to ethanol and the 2-3 butanediol (Kapdan and Kargi, 2006). Their H_2 yield on glucose is normally lower compared to that of *Clostridia*, however hydrogen production of anaerobic facultative bacteria (*Enterobacter aerogenes* and *Enterobacter cloacae* strain ITT-BY 08) have given interesting results (2.2 mol H_2/mol glucose; Kumar and Das, 2000).
Recently, hydrogen producing aerobic cultures such as *Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp. were also identified.
Hydrogen production performance of anaerobic thermophilic and hyperthermophilic organisms has also been investigated. Shin *et al.* (2004) reported *Thermoanaerobacterium thermosaccharolyticum* and *Desulfotomaculum geothermicum* strains producing hydrogen gas at high yield. Other bacteria, (*Thermococcus kodakaraensis* KOD1 isolated from a geothermal spring in Japan; *Clostridium thermolacticum* producing hydrogen from lactose at 58 °C; *Klebsiella oxytoca* HP1 isolated from hot springs) have been used by different researchers, as reported by Kapdan and Kargi (2006).

Compared with that of selected hydrogen-producing bacteria (pure culture), the hydrogen-producing ability of mixed cultured bacteria fed with complex organic substance is usually higher and the control and operation of the process easier (no medium sterilization is required). Therefore, beyond being a cheaper process, the use of mixed culture makes the microbial ecology more tolerant to stress and system imbalance. It depends on complex microbial interactions: the various species will grow in an interactive manner with commensalistic, ammensalistic, competitive and more complicated impacts of one specie to another. Thus the total resulting fermentative pathway will not correspond to what could be obtainable from individual species and each metabolic step will be

modulated by the overall interspecies available enzymatic activity (Lyberatos *et al.*, 2005).
Interestingly, Lin *et al.* (2003) studied the cooperation of hydrogen-producing fermentation bacteria in mixed culture with a batch test. Their results demonstrated a tight cooperation within the mixed culture, but on the other side, also demonstrated that the cooperation is conditional on the substrates: when fed with glucose (easily used by H_2-producing bacteria), the hydrogen-producing ability might be restrained because of the competition for the substrate between hydrogen-producing bacteria and other fermentation bacteria.
Indeed, the main disadvantage of the mixed culture is the possible predominance of non-hydrogen producing species such as methanogens, homoacetogens and lactic acid bacteria: to minimize this risk an initial pretreatment of the seed, together with the maintenance of environmental conditions unsuitable for the hydrogen consuming species, is usually required.

De Vrije and Claassen (2005) made an interesting overview of the different pure strains and mixed culture used in dark fermentative biohydrogen production tests, resumed in a table reported here (Table 1.4, Courtesy of De Vrije and Claassen, 2005) together with experimental conditions used, H_2 yields and H_2 production.

Tab. 1.4 Hydrogen yields and production rates by microorganisms as reported in the scientific literature (Courtesy of De Vrije and Claassen, 2005).

microorganism	conditions				substrate	H_2 yield mol/mol monosaccharide	H_2 production rate (maximal) mmol/h.L
	culture	*D*, h[1]	pH	T, °C			
Strict anaerobes							
Clostridia							
Clostridium sp. no 2	batch		6.0	36	glucose	2.0	23.9
	batch		6.0	36	xylose	2.1	21.7
C. paraputrificum M-21	batch		uncontrolled	37	GlcNAc[1]	2.5	31.0
C. butyricum LMG1213tl	continuous	0.222	5.8	36	glucose	1.5	21.7
Clostridium sp. no 2	continuous	0.18	6.0	36	glucose	2.4	7.1
	continuous	1.16	6.0	36	glucose	1.4	20.4
	continuous	0.96	6.0	36	xylose	1.7	21.0
Thermophiles							
Thermotoga maritima	batch		uncontrolled	80	glucose	4.0	10
Thermotoga elfii	batch		7.4	65	glucose	3.3	2.7
Caldicellulosiruptor saccharolyticus	batch		7.0	70	sucrose	3.3	8.4
Facultative anaerobes							
Enterobacter							
E. aerogenes E.82005	batch		6.0	38	glucose	1.0	21
E. cloacae IIT-BT 08 wt	batch		uncontrolled	36	glucose	2.2	
	batch		uncontrolled	36	sucrose	3.0	35
E. cloacae IIT-BT 08 m DM_{11}	batch		uncontrolled	36	glucose	3.4	
E. aerogenes E.82005	continuous	0.32	6.0	38	molasses	0.7	20
E. aerogenes HU-101 wt	continuous	0.67	uncontrolled	37	glucose	0.6	31
E. aerogenes HU-101 m AY-2	continuous	0.67	uncontrolled	37	glucose	1.1	58
Co-culture							
C. butyricum IFO13949 + *E. aerogenes* HO-39	continuous	1.0	5.2	36	starch	2.6	53
		HRT, h					
Mixed cultures from:							
- sludge compost	continuous	12	6.8	60	waste water sugar factory	2.5	8.3
- sewage sludge	continuous	6	5.7	35	glucose	1.7	29.6
	continuous	8	6.7	35	sucrose	1.7	26.2
- fermented soybean meal	continuous	8.5	6.0	35	glucose	1.4	8

[1]GlcNAc=*N*-acetyl-D-glucosamine

- *Genetic engineering strategies*

Different studies on hydrogen production by dark fermentation focused on artificially regulating and controlling bacteria metabolic pathways, aiming at enhancing hydrogen-producing efficiency at a microbial molecular level. The development of the following areas rouses the scientific community's interest:

- *Identification and isolation/selection of high-efficient hydrogen-producing bacteria.* Among the microbial H_2-producing species (*Bacteroides*, *Zymomonas*, *Clostridium*, *Fusobacterium*, etc.) some bacteria with peculiar characteristics were reported. For example, Ren *et al.* (2003) found a new genus of fermentative H_2-producing bacteria, including the strains *Rennanqilyf* 1 and

B49. The latter in particular had a good acid resistance (optimal pH under stirring cultivation: 3.9-4.2) and high H_2 production. Specific hydrogen conversion rate and hydrogen production rate of strain YUAN-3T, isolated by Xing *et al.* (2006), were 2.81 $mol_{H2}/mol_{glucose}$ and 27.6 $mmol_{H2}/g_{dry\ cell}$ h, respectively, and additionally this strain is the only auto-aggregative bacterium among the hydrogen-producers reported in literature up to date.

- *Regulation and control of enzyme genes involved in fermentative hydrogen production or in correlated metabolic pathways.* Activity and time of expression of hydrogenase, the last rate-limiting enzyme of hydrogen production pathway, directly affect the metabolism of hydrogen-producing bacteria, and thereby influence the production rate and yield of hydrogen (Liu *et al.*, 2008). Many researchers made studies about the expression level of hydrogenase and showed that its overexpression typically enhances the hydrogen-producing rate (Mishra *et al.*, 2004; Morimoto *et al.*, 2005). Also other enzymes can be regulated: for example Yoshida *et al.* (2005) performed the genetic overexpression of the formate hydrogen lyase (FHL) in *E. coli*, effectively resulting in a 2.8-fold increase in hydrogen productivity of the mutant strain compared with the wild type strain
- *Increased application of microbial molecular breeding and development of new techniques for breeding hydrogen-producing bacteria.* Presently, gene chip, microarray, real-time quantitative polymerase chain reaction, protein two-dimensional electrophoresis, multidimensional liquid chromatography, and surface plasmon resonance are playing a promoting role in microbial molecular biology and microbial molecular breeding (Liu *et al.*, 2008). However, some transformation and expression systems of fermentative hydrogen-producing microbes are still imperfect, and the study on molecular breeding aiming at

improving the hydrogen-producing fermentation pathways is still in the initial stages.

- *Hydrogen producing mixed microflora from natural sources: inoculum acclimation and methanogens control*

Zuo *et al.* (2005) used (pre-heated) river sediments as seed sludge to achieve anaerobic biohydrogen production. But this is just one of the different natural sources exploitable in a dark fermentative process. Previous authors demonstrated that H_2-producing consortia can be obtained from various environmental sources, such as soil, compost, sewage sludge and various fermented organic materials (Kyazze *et al.*, 2006; Li and Fang, 2007) and in Paper II we report interesting results from the preparation and use of both soil-inocula (a rice soil, a green urban soil and a vegetables-cultured soil) and anaerobically digested materials. All our works (Paper I, II and III) employ similar inoculum acclimation strategy.

Indeed, since dark fermentation has been shown to have great potential as applicable process to produce biohydrogen from a variety of organic materials, mixed cultures easily obtainable from natural sources and able to operate on non-sterile feedstock are required for future real implementation of this bioprocess. (Hawkes *et al.*, 2002). Lyberatos *et al.* (2005) state that a mixed culture with numerous microorganisms capable of degrading different organic compounds should be ensured in the initial inoculum, whose choice should be based on both the biomass used as substrate and the desirable products. Therefore the acclimation of a microbial culture is a very important process which brings significant changes to the microbial population and adapts the microbial to a specific substrate. A well acclimated heterogeneous culture is characterized by better performance, concerning the efficiency and the selectivity of the fermentation process, compared to the initial inoculum (Lyberatos *et al.*, 2005). For example, Liu (2008) and other authors (Radmann *et al.*, 2007; Yokoi *et al.*, 2002) report many operational advantages

using the "repeated batch cultivation technique", a well-known method for acclimating bacteria and enhancing the productivity of microbial cultures while simultaneously controlling the nutrients feed rate.

Nevertheless, in order to scale-up processes to make industrial production of biohydrogen economical, not only obtaining the inoculum from natural sources, but also avoiding loss of hydrogen through interspecies transfer - primarily to methanogens naturally occurring in non selected mixed cultures - is required. Three methanogens-inhibiting treatment methods have been reported so far, which are heat shock, pH control, and 2-Bromoethanesulfonic (BES) acid control. Most bio-hydrogen researchers use high temperature (range 75 °C - 121 °C) to inactivate hydrogenotrophic bacteria and harvest anaerobic spore-forming bacteria such as *Clostridium*: this process is pretty fast and its duration varies between 15 min and 2 h (Gavala *et al.*, 2006; Ntaikou *et al.*, 2010a; Oh *et al.*, 2003; Wang *et al.*, 2003a). In our works (Paper I, II and III) we typically heat shocked the inoculum at 100 °C for 1 or 2 h.

The pH control method is based on inactivating the methanogens maintaining them in a low pH (5 - 5.5) environment, on the other hand suitable for H_2-producing bacteria (Chang *et al.*, 2002; Oh *et al.*, 2003). Otherwise, BES ($C_2H_4BrO_3SNa$) is introduced as a specific methanogen chemical inhibitor but failures have also been reported about the use of this chemical in biologic fermentative systems, probably due to the high concentration used, far from the requirements of real situations (Lee *et al.*, 2009; Liu *et al.*, 2006).

- Reactor type and design optimization

Reactor design deeply influences the process performances through different aspects (the reactor microenvironment, the established hydrodynamic behavior, the contact between substrate and consortia, etc.), achieving substantial increases in hydrogen yield if well optimized.

Assuming that batch mode is more suitable for research purposes about fermentative hydrogen production (Ntaikou *et al.*, 2010a), industrially feasible process would work in continuous or semicontinuous mode and should exploit the well-established and commercial available AD technologies, currently used for wastewater treatment and biogas production. Among them, it is possible to distinguish three categories, based on the feedstock type:

- wet fermentation system: it fits to waste streams of less than 15% total solids, therefore solid waste streams are often diluted with recycled process water to form a slurry. The continuous stirred tank reactor (CSTR) is the most used reactor type to digest low solid waste streams (animal manure, sewage sludge, household waste, agricultural wastes, feces, urine, kitchen waste or mixtures of these substrates), offering simple construction, ease of operation and effective homogenous mixing as well as temperature and pH control. These digesters consist of a void recipient stirred by biogas recirculation, liquid recirculation or mechanical means (Figure 1.7). Mechanically stirred CSTRs were used in our continuous tests about hydrogen and methane production from anaerobic digestion of organic substrates/wastewaters (Paper II and III).

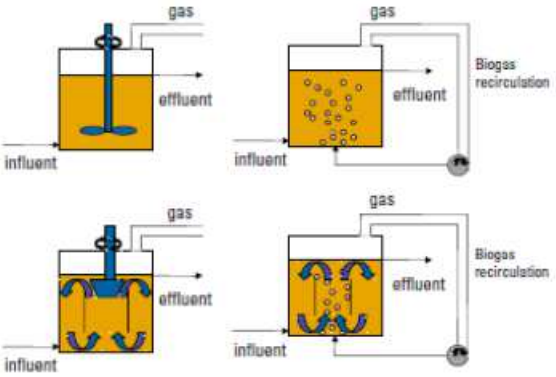

Fig. 1.7 Schematic diagram of a CSTR mechanically stirred (top) and stirred by biogas recirculation (bottom) (Courtesy of de Mes *et al.*, 2005).

CSTRs have the advantage of maintaining constant, homogeneous and well characterized process conditions, usually preventing stratification and formation of a surface crust and ensuring that solids remain in suspension. On the other hand, if process parameters are not correctly designed, bacterial washout may occur, thus decreasing the reactor performance.

- dry fermentation system: it fits to waste streams with a total solid percentage of more than 20% and to energy crops.
- high rate systems: they fit to wastewaters in high rate continuous flow. Reactors that allow high hydraulic loading rates without the washout of microorganisms could be divided into systems with fixed bacterial films on solid surfaces or into systems with a suspended bacterial mass, where retention is achieved through external or internal settling (like the contact digester or the upflow anaerobic sludge blanket - UASB - often adopted in hydrogen production tests).

Fixed-bed bioreactors containing a consortium of mesophilic bacteria have been reported to enhance the rate of hydrogen production to a greater extent than reported by other approaches (121 $mmol_{H2}/L_{digester}$ h; Chang *et al.*, 2002). In this case activated carbon as a support matrix allowed retention of the H_2-producing bacteria within the bioreactor.

In another study (Van Groenestijn *et al.*, 2002), hydrogen was produced in a high-rate bioreactor in the presence of hyperthermophilic bacteria, which formed a biofilm within an anaerobic trickling filter containing packing materials with a very high surface area. This resulted in the continuous flow of liquid-suspended biomass substrate through the filter, so that the biomass substrate, the H_2-producing bacteria and the resulting gas phase were in close proximity. The energy required to run this process was at least four times lower than the combustion value of the H_2 gas produced in such reactors (Van Groenestijn *et al.*, 2002).

Lastly, gas hold-up is one main problem for bioreactors: to mitigate the problem and to improve performance in terms of both the rate of hydrogen production and gas hold-up, tapered and rhomboidal bioreactors have been proposed. Gas hold-up was found to be reduced by 67% using a rhomboid bioreactor compared with a tubular bioreactor and high hydrogen production rate was achieved (75.6 $mmol_{H2/}L$ h; Kumar and Das, 2001).

- *Organic loading and hydraulic retention time (HRT)*

In batch tests in the mesophilic temperature range, it has been observed that the overall biohydrogen yield is a function of the organic load (OL, e.g. the amount of organic material added to a particular environment), with the highest yields obtained at the lowest OL (Van Ginkel *et al.*, 2001). Our work (Paper II) confirms this result, as increased glucose concentrations (1-7 g/L) caused a progressive decrease in H_2 yield (from 2.8 to 1.78 $mol_{H2}/mol_{glucose}$). The increase of substrate load to the system may cause higher levels of inhibitory metabolites and change chemical equilibrium, depressing further hydrogen production (Van Ginkel *et al.*, 2001; Zhang *et al.*, 2003).

This seems to be confirmed also in continuous culture, since comparing tests by different authors (all using glucose as substrate and adopting similar hydraulic retention time (6-12 hours)) the yield achieved at 30, 20, 7 and 3 g_{COD}/L were respectively 1.1, 1.7, 2.1 and 2.4 $mol_{H2}/mol_{glucose}$ (Fang and Liu, 2002; Kim *et al.*, 2006; Lin and Chang, 1999; Taguchi *et al.*, 1995). Again, Kataoka *et al.* (1997) found that increasing the glucose concentration from 5 to 10 g/L decreased H_2 yields from 2.0-2.3 to 1.4-2.0 $mol_{H2}/mol_{glucose}$. Therefore, Van Ginkel and Logan (2005a) assume that biohydrogen yields would be optimized for more dilute feeds and lower organic loading rates than those typically used in biohydrogen reactor studies.

Related to the OL, the HRT (hydraulic retention time) and/or the SRT (solid retention time) strongly affect the stability and the performance of a fermentation system and consequently the yield and selectivity of specific metabolic products. The HRT is defined as $\frac{V_r}{Q}$, where V_r is the active volume of the fermenter and Q is the influent flow rate. The SRT is defined as the mass of solids in the fermenter divided by the solids effluent rate and in CSTR reactor is equal to HRT. For experiments with pilot or laboratory scale reactors, HRT needs to be optimally selected and adapted to specific reactor design and metabolic products (Lyberatos *et al.*, 2005). In CSTR system, short HRTs (< 3 days) are generally used together with easily fermentable substrates, in order to wash out the slow growing methanogens (requiring more than approx. 3 days to grow) and to select for the acid producing bacteria (Chen *et al.*, 2001). Indeed, Ntaikou *et al.* (2010a) state that in CSTR a HRT of 12-36 h, depending on the substrate, provides complete conversion of carbohydrates and highest hydrogen yields. However, it is also true that too short HRT could lead to bad hydrolysis of organic wastes (Han and Shin, 2004a) or to pH unbalance and thus to lower H_2 production. Our considerations about HRT influence on maximizing biohydrogen production and process stability are reported in Paper I, where we investigated a wide range of HRT (1-4.5 d) associated with fruit-vegetable waste and swine manure mixture fermentation.

- *Temperature*

Temperature is an important factor for microbial activity, which regulates the metabolic and reproduction rates of microorganisms. There are mainly three temperature intervals considered valid for hydrogen production by anaerobic fermentation: the psychrophilic (Temperature range 0-20 °C, optimum 15 °C), the mesophilic (Temperature range 15-45 °C, optimum 37 °C) and the thermophilic (Temperature range 45-75 °C, optimum 55 °C). Moreover, extreme

thermophiles or hyperthermophiles H_2-producing microorganisms exist, whose growth optimal temperature is above 65 °C and 80 °C, respectively (Levin *et al.*, 2004).

Psychrophilic digestion is not commonly used, except for some applications in the northern countries of the world, mesophilic temperature is the most common range adopted, while thermophilic digestion has been reported to have several advantages over the others, such as higher reaction rates and pathogen-killing effect (Meulepas *et al.*, 2005). Therefore, we generally conducted our experiments (Paper I, II and III) in thermophilic conditions (55 ± 2 °C). However, interesting studies reported that extreme-thermophilic fermentation can further minimize the contamination by pathogens and hydrogen consumers (Liu, 2008), achieve higher hydrogen total production and production rate than mesophilic hydrogen fermentation (van Groenestijin *et al.*, 2002), and reach the theoretical maximum yield of 4 $mol_{H2}/mol_{glucose}$ (van Niel *et al.*, 2002).

- *pH*

The hydrogen-producing bacteria are quite sensitive to pH fluctuation because pH change may result in the change of their metabolic pathway: medium pH affects enzyme activity in microorganisms (since each enzyme is active only in a specific pH range) and thus hydrogen production yield, biogas content, type of the organic acids produced and specific hydrogen production rate. Under not optimal pH, the hydrogen fermentation process may prolong the lag phase or shift to other pathways, such as solvent production (Cheng *et al.*, 2002; Liu, 2008; Temudo *et al.*, 2007). Very low initial pH of 4.0 - 4.5 causes long lag periods such as 20 h (Khanal *et al.*, 2004; Liu and Shen, 2004), while initial pH of 9.0 may decrease the lag time but gives lower biohydrogen yield and higher risk of hydrogen consuming activities (Zhang *et al.*, 2003).

The optimal pH for ethanol-type fermentative bacteria ranges from 4.0 to 4.5, while the pH range for the maximum hydrogen yield in

metacetonic acid-type fermentation is between pH 5.0 and 6.0 (Gomez *et al.*, 2006; Kapdan and Kargi, 2006; Liu *et al.*, 2006).

However, fermentative bacteria own balancing and regulating abilities and can withstand pH also different from their optimum. For example for the (extreme)thermophilic acid-type hydrogen fermentation the optimum pH range was reported to be between 6.8 and 8.0 (Liu, 2008; Van Niel *et al.*, 2002; Yokoyama *et al.*, 2007) or oppositely around pH 4.5 (Shin *et al.*, 2004).

For real, without pH adjustment of the media, many studies report that at the end of anaerobic hydrogen production via metacetonic acid-type fermentation, the medium pH shifts away from its optimum and it can reach values between 4.0 and 4.8, regardless of initial pH (Liu *et al.*, 2003; Liu and Shen, 2004; Morimoto *et al.*, 2004; Yokoi *et al.*, 2001). Indeed, in an unbuffered system, the pH decreases due to production and accumulation of organic acids which deplete the buffering capacity of the medium. This, often caused by system overloading, may inhibit hydrogen production by affecting the activity of iron containing hydrogenase enzyme (Kapdan and Kargi, 2006; Khanal *et al.*, 2004).

Therefore, medium pH adjustment through addition of chemicals (acid or base) is often required to maintain the pH around its optimum. On the other hand, this approach may not be optimal for large-scale transfers, and when looking for full-scale applications different strategies may be considered for maintaining acceptable chemical equilibrium in fermentation broth.

So, in our studies (Paper I and III) enhancement of the buffer capacity of the system to avoid the pH drop and maintain it around its optimum, was reached by mixing the fermentable substrates with alkalinity rich wastewater (such as swine slurry). Even effluents recycled from AD process may be used in this strategy.

- *H_2 and CO_2 partial pressure and gas sparging*

The accumulation of hydrogen and carbon dioxide can lead to repression of H_2 production (due to end-product inhibition) and to formation of more reduced products (Classen *et al.*, 1999; Nath and Das, 2004).

In anaerobic digestion with mixed anaerobic cultures, the accumulation of hydrogen is normally balanced by rapid hydrogen consumption by methanogens, resulting in little net hydrogen accumulation in the system (Mahyudin *et al.*, 1997). On the contrary, in Dark Fermentation hydrogen must be produced and accumulated without being consumed. However, if hydrogen concentrations increase over some fixed limits (pH2 of >50 kPa at 60 °C, >20 kPa at 70 °C, and >2 kPa at 98 °C), H_2 synthesis decreases and metabolic pathways shift towards production of more reduced substrates, such as lactate, ethanol, acetone, butanol or alanine (Levin *et al.*, 2004).

Therefore, the system used for Dark Fermentation must be designed to both remove hydrogen before it leads to repression of its production and to prevent interspecies hydrogen transfer leading to methanogenesis (Mahyudin *et al.*, 1997; Tanisho *et al.*, 1998).

Therefore gas sparging has been found to be a useful technique to reduce hydrogen partial pressure (*pp*) in the liquid phase and enhance H_2 yield.

In a study by Mizuno *et al.* (2000), it was observed that the specific hydrogen production rate increased from 1.446 $mL_{H2}/min\ g_{biomass}$ to 3.131 $mL_{H2}/min\ g_{biomass}$ under nitrogen sparging conditions. With N_2 sparging at a flow rate approximately 15 times the hydrogen production rate, the hydrogen yield was 1.43 $mol_{H2}/mol_{glucose}$. This meant an increase in hydrogen yield due to nitrogen sparging of almost 50%. A report by Tanisho *et al.* (1998) revealed that sparging with argon results in an increase of residual NADH, which might be expected to increase hydrogen production. A hollow fiber/silicone rubber membrane effectively reduced biogas partial pressure in a

dark fermentation system, resulting in a 10% improvement in the rate of hydrogen production and a 15% increase in H_2 yield (Liang *et al.*, 2002).

For real, the advantages of gas sparging are also connected to the CO_2 removal, because its accumulation may decrease the yield of hydrogen due to electrons consumption for succinate and formate synthesis via CO_2, pyruvate and NADH (Das and Veziroglu, 2001). Tanisho *et al.* (1998) stated also that CO_2 partial pressure may have higher inhibition effect to the dark fermentative hydrogen production than H_2 *pp*. Several attempts to remove CO_2 have been made either by inert gas sparging to drive out hydrogen and carbon-dioxide from the reactor or by employing other membrane-based processes. Not only inert gases like argon but also hydrogen itself was effective in the removal of CO_2. H_2 sparging may also be economical because the production plant won't need to separate the mixed gas if the produced hydrogen will be used as the removing gas (Tanisho *et al.*, 1998). Thus, Tanisho *et al.* (1998) increased the hydrogen yield from 0.52 up to 1.58 $mol_{H2}/mol_{glucose}$ by the combined effects of CO_2 removal and conditions of sufficient nitrogen source. Table 1.5 (Courtesy of Nath and Das, 2004) provides an excellent overview of the state-of-the-art technologies for hydrogen removal from a reaction system.

Tab. 1.5 Different approaches of hydrogen removal. (Courtesy of Nath and Das, 2004).

Methods	Advantages	Disadvantages	References
N_2 sparging	50% increase in H_2 yield compared to no sparging	Sparging gas should be free of CO, otherwise it could inhibit hydrogenase	Mizuno et al. 2000
N_2/Ar sparging	Simultaneous lowering of H_2 partial pressure	Too much sparging dilutes H_2 and creates problems in separation	Hawkes et al. 2002
Sparging with fuel cell exhaust gas	Under investigation	Feasibility not yet reported	Hawkes et al. 2002
Hollow fiber submerged silicone membrane	Effectively reduces biogas partial pressure in the reactor	Reduced efficiency due to biofilm build-up	Liang et al. 2002
Steam stripping by evaporation at a large surface	Separation of steam from H_2 is much easier. (by condensation)	May lead to cooling of fermentation broth in thermoreactor	Van Groenestijn et al. 2002
Stripping H_2 with recirculation gas	Can be carried out in reactor having either liquid or gas as continuous phase	High energy consumption. Problem of creation of gas-liquid interface	Van Groenestijn et al. 2002
Pd-Ag membrane reactor	High hydrogen selectivity. 85–90% of H_2 can be removed	CO contamination affects membrane performance	Nielsen et al. 2001
Synthetic polyvinyl-trimethyl silane membrane	High H_2 selectivity	–	Teplyakov et al. 2002

- *Nutrients and metal ions requirements*

In general, hydrogen production in AD has lower requirements of macro and micro nutrients than aerobic processes, due to the process lower microbial biomass yield. Of course, carbon, nitrogen and phosphorous are fundamental, with nitrogen that is especially important not only for bacterial growth and multiplication but also because the digestion of nitrogenous compounds could contribute to the pH buffering of the bioprocess by releasing ammonium cations (Liu and Shen, 2004). Even the balance between the nutrients is fundamental, such as the C/N ratio which is reported to deeply affect the hydrogen productivity and the process stability (Tanisho *et al.*, 1998). Lin and Lay (2004) demonstrated that increasing the C/N ratio from 40 to 47, hydrogen production in mesophilic hydrogen fermentation from sewage sludge was 5 times higher.

Among the micronutrients, the most influencing elements are sulfur, vitamins and traces of minerals: many function of anaerobic bacteria are strongly dependent on the availability of trace elements, since they form part of the active sites of several key enzymes (Meulepas *et al.*, 2005). In particular, iron (Fe) shortage could influence the growth, metabolism, and hydrogen-producing ability of B49 (an anaerobic bacterium strain). Ren *et al.* (2003) suggest that adding Fe^{2+} could increase hydrogen enzyme activities (such as NADH-Fd reductase) and consequently enhance bacteria hydrogen-producing ability. Indeed, ferrodoxin (Fd) is an iron-sulfur protein which requires Fe and functions primarily as an electron carrier, being involved in pyruvate oxidation to acetyl-CoA and CO_2 and in proton reduction to molecular H_2. Fe is involved also in the control of lag phase (increased by high iron concentrations, such as 100 mg_{Fe}/L added in batch hydrogen production by Liu and Shen, 2004) and metabolic pathways (butyric acid-type fermentation may turn into ethanol-type by adding Fe; Wang *et al.*, 2003b).

Another important influencing factor is magnesium (Mg^{2+}): its shortage may limit the growth anabolism of hydrogen-producing fermentative bacteria (such as B49), and its hydrogen-producing ability. Addition of Mg^{2+} is reported to promote the growth of ethanol type hydrogen-producing fermentative bacteria and enhance their hydrogen-producing ability (Liu *et al.*, 2008).

- *Toxicants*

Toxic compounds can be found in the feedstocks or can be produced by microorganisms converting non inhibitory substances to inhibitory ones. Among the toxicants, it is possible to find inorganic substances (heavy metal cations, hydrogen sulphide, salts and ammonia at relative high concentrations) and organic compounds (polyphenols, furfural and hydroxyfurfural compounds). Their toxic effect may be even increased by other process factors: for example ammonia inhibiting effect is higher with higher pH, since this makes easier the release of free ammonia into the medium (Meulepas *et al.*, 2005). As for the inhibitory organic compounds, they are commonly generated during physicochemical or biological pretreatment of lignocellulosic biomass. Also wastes originating from different agricultural products typically contain polyphenolic compounds, even frequently found in animal manure. These toxicants presence could be a big issue also for a second stage fed with the effluent of a first dark fermentative stage (see Chapter 4). Indeed, phenol, indole and benzene, with and without substituents, were found by us in the first stage effluent of a two-stage H_2-CH_4 producing reactor, deeply studied and characterized in Paper III. Lastly, also oxygen could be considered a toxicant for obligate anaerobic microorganisms (see the following section) and in addition any highly oxidized material, as nitrate or nitrite, can exhibit inhibition.

- *Anaerobic conditions and reducing agents*

Among the fermentative hydrogen-producing microorganisms, obligate anaerobes such as *Clostridium butyricum* are extremely sensitive to oxygen and their hydrogen-producing activities are completely inhibited by the presence of a very slight amount of oxygen in the feeding medium (Yokoi *et al.*, 1995). Therefore reducing agents such as argon, nitrogen, l-cysteine and the same hydrogen gas may be used to remove trace amounts of oxygen present in the medium and to decrease redox electric potential. However, the use of such reducing agents is relatively expensive, and therefore uneconomical for industrial biological production of H_2.

Alternatively, *Enterobacter aerogenes* or other facultative anaerobes, which have the ability to survive and work in the presence of slight amount of oxygen within the bioreactor, may be exploited. Yokoi *et al.* (1998) suggested the use of a mixed culture of strict and facultative anaerobic bacteria where *E. aerogenes* rapidly consumes oxygen, thus recovering immediately strict anaerobic condition optimal for the more performing *Clostridium* bacteria.

Chapter 2

Biomasses for hydrogen production via Dark Fermentation

Biomass is considered an intrinsically safe and clean material, with unlimited availability and high potential to be used as a renewable source for the production of energy and alternative fuels, new materials for technical applications and organic materials and chemicals. In particular, biomass can be defined as "the biodegradable part of products, waste and residues from agriculture (including vegetable and animal substances), forestry and related industries, as well as the biodegradable fraction of industrial and municipal waste" (Italian Legislative Decree 29/12/03, n. 387 - Implementation of directive 2001/77/CE on the promotion of electricity produced from renewable energy sources in the internal electricity market).

On the basis of the increasing biomass exploitation by emerging technologies, assumptions have been made about the capability of

biomasses to contribute for 15% of the total world energy demand in 2020. Calculations based on an optimistically estimated maximal yield of 50 ton/ha for agricultural biomasses, indicate that an amount of 50 Gton biomass/year (80% non-food biomass, 10% forestry and 10% waste streams) could be available for non food applications in 2040 (Okkerse and van Bekkum, 1999).

However, in order to really compete with fossil fuel-based energy technology without any tax support, biomass-based energy systems must achieve feedstock and processing advantages over fossil fuels. Unfortunately, nowadays biomass energy production has costs that are inherently high compared with the gas/oil/coal-fired electricity generation processes, because:

- biomass fuels have low bulk density, they are expensive to gather, process, transport and handle;
- biomass power generators are smaller than conventional generators, which makes bioenergy generation economically disadvantageous;
- biomass energy technology is not as advanced and integrated with the needs of the society as those oil/natural gas/coal -based (Akay *et al.*, 2005);
- whereas the oil-based chemical technology for converting fossil feedstocks into a variety of useful products is very efficient and mature, the technology for converting agricultural raw materials is still in its infancy (Soetaert and Vandamme, 2005).

At the present, energy from biomass can be produced basically in two ways: chemical decomposition through thermal processes and biological conversion. Thermal processes (incineration in excess of oxygen, pyrolysis and gasification) have the general disadvantage of causing atmospheric pollution, unless costly purification of the effluent gases is applied.

Thus, a valid alternative are the biological processes, which essentially are anaerobic fermentative processes with production of ethanol, methane and hydrogen (Lyberatos *et al.*, 2005). As shown in

chapter 1, medium and long term strategies, from bioprocess intensification and miniaturization to genetic engineering of microorganisms and plants, have been developed for biologic biomass conversion and new programs have been started in order to harvest the benefits of the bio-based economy and to enhance the use of biomass for energy and chemicals. Still, the cost and the retrieval of substrates (biomasses) to be used in fermentative process is one of the main expenditure items.

A report of ITABIA (2008) (Table 2.1) summarized the quantity of overall available biomass in Italy every year, considering different organic residues, animal manure and dedicated energy crops, amounting to 24-30 Mtoe (millions of equivalent tons of oil) per year. This study stated also that the actual availability of biomass in Italy, regardless of collection and supply problems, is about 80% of potential availability, thus corresponding to 19-24 Mtoe/year. According to Coldiretti association, this could guarantee a saving of 10-12 millions of tons per year in oil consumption, with a simultaneous reduction of CO_2 emissions of 30 million tons.

Tab. 2.1 Million of equivalent tons of oil (Mtoe) yearly available from organic matter in Italy (Courtesy of ITABIA, 2008).

	Mtoe/Year
1. RESIDUES FROM	
AGRICULTURE AND AGRO-INDUSTRY	5
FORESTRY AND WOOD INDUSTRY	4.3
URBAN SOLID WASTE	0.3
ANIMAL BREEDING	10-12
2. FIREWOOD	2-4
3. ENERGY CROPS (POTENTIAL)	3-5
4. TOTAL	**24-30**

Making specifically reference to renewable hydrogen production, this amount of available biomass would have the potential to make

the biohydrogen-based technologies cost-competitive to those natural gas-based (by now cheaper).

The Department of Energy of the United States has recently set ambitious goals about biomass conversion into hydrogen by dark fermentation (Logan, 2004), e.g.

- to reach 50% conversion efficiency for hydrogen production from biomass;
- to reduce hydrogen costs from \$6 to \$1.50/$kg_{biomass}$;
- to extract high-purity hydrogen from biomass at relatively low cost of \$2.60/$kg_{biomass}$ in short-term period.

However, waiting to fulfill these requirements economic competitiveness of biohydrogen/bioenergy production could be increased through biomass integration in relatively small scale (0.1-20 MWe) applications, aiming at local needs satisfaction with simultaneous environmental impact reduction.

Therefore, specific attention must be paid to biomass chemical composition, because, as stated previously (see chapter 1), biomasses rich in sugars and/or complex carbohydrates are the preferred ones for fermentative hydrogen production, potentially achieving a 20 times higher production than fat-rich wastes (fat meat, chicken skin...) and protein-rich wastes (Lay *et al.*, 2003; see Table 2.2, Courtesy of Show *et al.*, 2011).

Tab. 2.2 Comparison of maximum hydrogen yields of different substrates (Courtesy of Show *et al.*, 2011).

Substrates	Constituents	Seed sludge	Hydrogen yield conversion (L H2/kg VS)	Conversion efficiency (%)	References
Carbohydrates	Pure glucose	Thermotoga maritima	497.8	100	Shroeder *et al.* 1994
	Cellulose	Sludge compost	298.7[a]	60	Ueno *et al.* 1995
	Starch	Thermococcus kodakaraensis KOD1	414.4[b]	83.25	Kanai *et al.* 2005
Proteins	Peptone	UASB sludge inhibited by chloroform	33.6	6.75	Liang *et al.* 2001
	Egg	Digested sludge	7.07	1.42	Okamoto *et al.* 2000
	Lean meat	Digested sludge	7.68	1.54	Okamoto *et al.* 2000
Lipids	Chicken skin	Digested sludge	10.1	2.05	Okamoto *et al.* 2000
	Fat	Digested sludge	11.2	2.23	Okamoto *et al.* 2000

[a]Calculated from the reported value of 2.40 mol H2/mol hexose

[b]Calculated from the reported value of 3.33 mol H2/mol hexose (starch)

In the following sections the types and the availability of the exploitable actual biomasses will be explored in details: section 2.1 focuses on dedicated agro-zootechnical and lignocellulosic (from agriculture, forests, energy crops) feedstocks; section 2.2 on heterogeneous wastes and wastewaters, such as municipal solid waste (MSW) and its organic fraction (OFMSW), sewage sludge and industrial wastes.

2.1 Agricultural and livestock biomasses

Yearly, a total of about 170 Gton biomass is worldwide produced through photosynthesis in green plants and the energy value of this annual production (2.74×10^{19} Btus - British thermal units -) is eight times as much as the annual world energy consumption (3.4×10^{18} Btus) (Lyberatos *et al.*, 2005). However only 3.5% (6 Gton) of the biomass produced is being cultivated, harvested and used. Of this, 62% (3.7 Gton) is consumed for the production of food, 33% as fuels for energy and housing and just approximately 0.3 Gton (5%) are used by non food industry (Eggersdorfer *et al.*, 1992).

The word "energy crops" refers therefore to that 33% of biomass cultivated for being further exploited (either whole or part of it) as feedstock for energy production, i.e. energy gain through combustion or biotransformation to biofuels. Ntaikou *et al.* (2010a) state that the sustainability of such processes can only be assured if:

- the crops are produced at low cost, thus with minimum nutrient and water requirements;
- the crops are resistant to environmental stresses;
- the crops are highly biomass yielding;
- the crops have high sugar and/or carbohydrates' content and low lignin content (specifically for hydrogen production via dark fermentation).

If employed in wet processes, such as in anaerobic digestion, also the water content is one of the key parameters for the choice of a specific crop. Indeed, water is important for biological activity, since nutrients must be dissolved in water before they can be assimilated, and it enhances the mobility of microorganism, improving the mass transport and the penetration/diffusion of microorganisms throughout the substrate, thus facilitating the digestion process (Meulepas *et al.*, 2005). Moreover a water content usually higher than 35% (together with a C/N ratio in the range of 20-30) make biomasses fit better to bioprocessing wet technologies than to direct incineration, where supplemental fuel would be required, proportionally to the water content. Moisture, ash content and gross calorific values (CV) of different solid biomass feedstock are given in Table 2.3 (Nath and Das, 2003).

Tab. 2.3 Moisture and ash content and gross calorific value of different biomass feedstock (Courtesy of Nath and Das, 2003).

Biomass	Moisture (%)	Ash (%)	CV (MJ/kg)
Bagasse	50	1–2	9.2
Bagasse pith	40	2	7.5–8.4
Spent bagasse	40	10	12.5
Sawdust	35	2	11.3
Rice husk	10–15	15–20	12.6–13.8
Rice straw	6	16	14.4
Deoiled rice bran	16	16	11.3
Coffee husk	11–14	2–5	15–17.5
Peanut shell	10	2–3	16.75
Coconut shell	10	1	18.8
Coir pith	8	15	16.75
Cotton stalk	7	3	18.4
Soya straw	7–8	5–6	15.5–15.9

However, the main criterion driving the adoption of a specific biomass, especially for biohydrogen production, is its chemical composition: energy crops can be divided in sugar based crops (e.g. sweet sorghum, sugar cane and sugar beet), starch based crops (e.g.

corn and wheat), and lignocellulose based crops, including herbaceous (e.g. switch grass and fodder grass) and woody crops (e.g. Miscanthus and poplar). These crops can be processed by means of so-called biorefineries into relatively pure carbohydrate feedstocks, the primary raw material for most fermentation processes, or directly employed in fermentative hydrogen production processes. Therefore, the exploitation of lignocellulosic raw materials, consisting of tightly bound lignin, cellulose and hemicellulose, for hydrogen production via fermentation depends on the capability of exploiting cellulose and hemicellulose and simultaneously avoiding the lignin constituent. Indeed, the bonding in lignocellulose resists mobilization, as lignin is not degraded under anaerobic conditions and is often inhibitory to microbial growth (De Vrije and Claassen, 2005). In view of producing cheap feedstocks for dark hydrogen fermentation from lignocellulosic biomasses, development of cost effective pretreatment methods with a low energy demand must be studied.

Several energy crops (mainly starch- and sugar-based) have already been used in laboratory-scale experiments, such as maize, rye, Jerusalem artichoke, oat, sunflower, triticale, rape and wheat (Ahrens and Weiland, 2004) and Table 2.4 (courtesy of Ntaikou *et al.*, 2010a), shows the maximum hydrogen production and yield achieved via dark fermentation of different energy crops, with noticeable results from pretreated Miscanthus.

In our Paper II, Maize silage showed a relatively interesting biohydrogen production potential of 118 ± 2 NL_{H2}/kg_{VS}, almost the half of that of the more promising market bio-wastes and organic fraction of municipal solid wastes.

Tab. 2.4 Hydrogen production via dark fermentation from different energy crops (Courtesy of Ntaikou *et al.*, 2010a).

Crop	Microorganism	Operation mode	Maximum H_2 production rate (LH_2/l/day)	Maximum H_2 yield (mol H_2/mol cons. hexose)
Miscanthus (pretreatment: mechanical and NaOH)	*Thermotoga elfii*	Batch	–	1.1[a]
Wheat starch	Mixed mesophilic cultures	Continuous	3	1.26
Sugarbeet juice	Mixed mesophilic cultures	Continuous	2.2[b]	1.9
Corn starch	Mixed mesophilic cultures	Continuous	2.57	0.51
Sweet sorghum extract	Indigenous microbial mesophilic culture	Continuous	8.52	0.86
Sweet sorghum stalks	*Ruminococcus albus*	Batch	–	3.15 (59 l/kg wet biomass)
Sweet sorghum extract	*Ruminococcus albus*	Batch	–	2.61
Ryegrass	Mixed mesophilic cultures	Continuous	6	82[c]
Sweet sorghum	*Caldicellulosiruptor saccharolyticus*	Batch	–	1.75 (30.17 l/kg dry biomass)
Sugar beet extract	*Caldicellulosiruptor saccharolyticus*	Batch	–	–
Barley grains	*Caldicellulosiruptor saccharolyticus*	Batch	–	–
Corn grains	*Caldicellulosiruptor saccharolyticus*	Batch	–	–
Miscanthus (pretreatment: NaOH, $Ca(OH)_2$)	*Thermotoga neapolitana*	Batch	13.1[d]	3.2
Miscanthus (pretreatment: NaOH, $Ca(OH)_2$)	*Caldicellulosiruptor saccharolyticus*	Batch	12.6[d]	3.4

[a] mol H_2/mol consumed sugars

[b] ml/min l

[c] ml H_2/g dry mass

[d] mmol H_2/l h

As for animal (livestock) residues employment in biohydrogen production via fermentation, due to their chemical characteristics (alkaline pH, no carbohydrate content,...) they are commonly not suitable for this process. Only a few studies have addressed the potential use of swine manure as feedstock for biohydrogen production and when used as a single substrate alone, very little biohydrogen could be recovered from fermentation both at mesophilic temperatures (Wagner *et al.*, 2009), as well as at hyperthermophilic temperatures, with production yields lower than 4 L_{H2}/kg_{VS} (Kotsopoulos *et al.*, 2009). Our study (Paper II) confirmed low biohydrogen production potential of swine manure, achieving just 14 ± 1 NL_{H2}/kg_{VS}.

On the other hand, hydrogen yield as high as 200 L_{H2}/kg_{hexose} (Wu *et al.*, 2009; Zhu *et al.*, 2009) were obtained when swine manure was

added to glucose, reinforcing the hypothesis that livestock residues are a suitable co-substrate to be fermented by a mixture culture in addition to a carbohydrate-rich, promptly hydrolysable material.

Speculating on full scale hydrogen production via dark fermentation, this co-digestion strategy seems particularly advantageous, as demonstrated nowadays in Europe conventional AD process plants which hardly use agricultural crops alone, while co-digest them with manure or other organic wastes because of the positive influence of manure as source of essential trace elements and buffering substances for the biogas process (Holm-Nielsen and Al Seadi, 2004).

From an applicative point of view, over 65% of the biogas plants now operating in Germany utilize energy crops and crop residues in co-digestion with manure (Weiland *et al.*, 2003). Eurobserv'ER (2010) reported that the introduction of energy crops in co-digestion strategies, made the European biogas production increase and, as a consequence, made the electric energy production via biogas employment in cogeneration jump forward of about 20.5% between 2006 and 2007. This is especially true for UK and Germany, among the EU member countries, which have been playing the leading role because of their renewable energy policies providing incentives for farmers producing energy with small digestion units.

The same can be said for Italy, where, as indicated by ITABIA (2008), nowadays crops dedicated to energy production (competing with crops planted for food or industrial purposes) are limited to a few thousand hectares of sunflower, soy and rapeseed for biodiesel and other few thousand hectares of rapid growth poplars (Short Rotation Forestry, SRF) located in Northern Italy. The Italian agro-energetic system interest in biogas production by means of anaerobic digestion of agricultural biomasses is focused, on one hand, on the chance of giving integration to farmers' income and, on the other, on the significant economic and social role that AD can play as it

effectively can contribute to mitigate and even solve the environmental issues connected to the high concentration of livestock production units that can be found in some Italian Regions (e.g. Lombardy). The same remarks would be valid for biohydrogen production via Dark Fermentation.

By an analysis of Italian territory, ITABIA estimated that approximately 500,000 - 600,000 ha of arable land may be used to grow energy crops for bioenergy production, and that about 100,000 ha of marginal land may be used to grow low input energy crops that are ideal for both producing lignocellulosic biomass and biofuels. Overall vegetable production (lignocellulosic, oleoplants, sugary plants, etc.) can be estimated to be about 13-16 million tons (of fresh matter)/year, corresponding to 4-5 Mtoe/year of primary energy.

In consideration of animal manures, in Italy the pig industry alone consists of 9.5 millions animals and is mainly concentrated in highly specialized districts. It can be estimated that in the Po Valley area, more than 10 million tons of swine manure are produced yearly on a territory of about 5000 km^2. As a whole, 330 million tons of liquid wastes are produced every year, but only a part of it is used for anaerobic digestion, even if no other alternative competitive uses exist (apart for the limited share of liquid waste that can be spread on the fields) and if this is potentially a cause of environmental and societal problems, when not properly managed. On the contrary, as reported by the ITABIA report (2008), in Germany and The Netherlands there is a liquid waste stock exchange where trading rates range from 1.5 Euro/t within 5 km from the producing farm to up to 5 Euro/t beyond 5 km.

So, similarly to other Countries and considering the huge amount of animal slurries, crops and their residues (223 million tons/year, altogether; Piccinini - CRPA, 2008) yearly available in Italy, co-digestion of these wastes for biogas or biohydrogen production via fermentation is an advisable strategy for our Country.

However, despite the good biogas and biohydrogen productions guaranteed by energy crops, the continuously rising food prices, the sustainability doubts and the energy-equation challenges have recently led to a backlash against their use as feedstocks for biofuels generation. In particular a food-vs-fuel debate took place, since in many countries huge agricultural areas have been turned into feedstock "industries" for the production of chemicals, transport biofuels and energy (Ntaikou *et al.*, 2010a; 2010b).

Therefore, the second generation biofuels, produced by feedstocks that are not competitive to edible crops, such as wastes and residues, can be a valid solution to the "energy deviation" from the plants primary function of supporting human dietary needs. Substrates used for "second generation hydrogen" will be further discussed in the following section.

2.2 Organic residues and wastewaters

Wastes from agricultural and municipal processes should be the preferred biomass for energy production. Indeed, if the biomass employed in the bioprocesses would preferentially be derived from wastes and discarded residues, this will not increase the pressure on natural habitats or the conflicts with the global food availability and the preservation of biodiversity.

Nowadays unused biomasses are mainly burnt, land-filled or accumulated as excess biomass, potentially leading to leachates, greenhouse gases emission, soil and water contamination and so on. Moreover, the disposal of wastes is already an economic burden on communities and industries.

So, creating a marketable product (biofuel) from wastes would immediately make money by both reducing waste treatment costs and recovering energy (in form of methane or hydrogen) and/or valuable materials from their processing. An example about how much energy could be theoretically available in wastewaters is

reported by Logan (2004), considering the United States wastewaters production:

- the organic content in wastewater produced annually by humans is equivalent to 0.11 quadrillion of Btus (British thermal units) and worth $2 billion (assuming 330 million people producing 230 L $_{wastewaters}$/day, 300 mg $_{biological\ oxygen\ demand\ (BOD)}$/L $_{wastewaters}$, and 3.5 kcal/g $_{BOD}$);
- animal wastewaters have the potential for energy harvesting of an additional 0.3 quad. Btus;
- food processing wastewater, the most readily available source because of its high sugar content and low indigenous bacterial concentration, could have an overall energy content of 0.1 quad. of energy (assuming the exploitation of 5% of the total U.S. food industries wastewaters, having an average organic matter content of 2 g $_{chemical\ oxygen\ demand\ (COD)}$/L $_{wastewaters}$).

Logan (2004) stated also that, in order to compete with current electric power plant costs, a wastewater source annually containing 0.1 quad. would have to be harvested for less than $3.3 billion. However, higher costs could be tolerated in the United States if they are included in the $45 billion already needed over the next 20 years for wastewater treatment infrastructure or if they are used to reduce annual expenditures in the $25 billion wastewater industry.

To further maximize economic competitiveness of bioenergy production processes toward common fossil energy strategies, wastes should be digested on site, so that their exploitation by small-scale power generation plants can compete with fossil fuel-based energy production, advantaged for example by feedstock logistics (no transportation from one place to another).

An interesting report by ITABIA (2008) assessed the italian availability of various (biomass) residues, mainly from the five most relevant sectors: agriculture, forestry, agro-industry, wood industry and urban waste. The estimated total quantity of organic residues and

by-products produced in Italy every year amounts to more than 25 million tons of dry matter (see Figure 2.1). Unfortunately only a part of it can be used nowadays, due to:

1) Competition with the non-energy uses of the biogenic matter;

2) Problems with collection of materials and their subsequent supply to the energy conversion plant.

Fig. 2.1 Total annual quantity (k.ton of dry matter per year) of biomass residues in Italy.

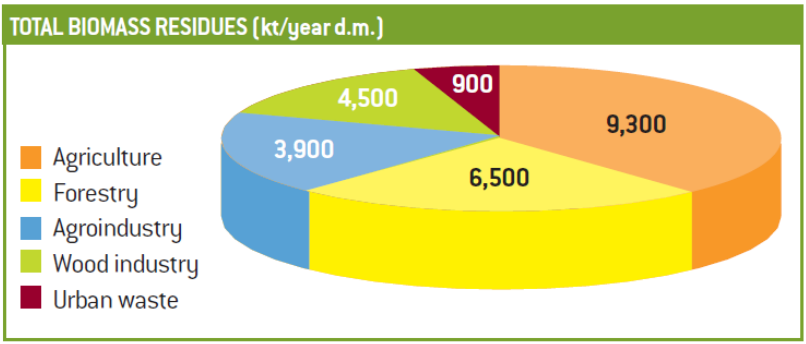

Table 2.5 presents detailed esteems for specific residues availability in Italy. Specifically:

- *Agricultural residues*

Lignocellulosic wastes (straw, stalks, prunings, etc.) from agriculture sector (herbaceous and woody plants) have an estimated available quantity (excluding the share of existing but unusable residues) that amounts to approximately 9.3Mt/year of dry matter;

- *Forestry biomass*

Forestry residues that can be used for energy are estimated by analyzing actual firewood production: firewood production in Italy today amounts to approximately 2.2. Mt/year of dry matter (4.5 Mt/year of wet matter) but a significant increase in resources (4.3 Mt/year in d.m.) for the wood-energy supply chain would be required in order to reach the calculated 6.5 Mt/year from today's production.

- *Industrial residues*

The overall availability of industrial residues, expressed as dry matter, amounts to around 8.4 Mt/year, of which 3.9 Mt come from agro-industry and 4.5 Mt/year from the wood industry;

- *Urban wastes*

Today, in Italy only 8% - 10% of the wastes of urban origin is used as fuel, in compliance with Legislative decree 152/06. Indeed:

- Recycling produces almost 0.9 Mt/year of wet matter, which means about 100,000 t/year of dry matter.
- The maintenance of public greeneries yields over 9 Mt/year of wet matter, which means about 380,000 t/year of dry matter (ITABIA, 2008).

The organic fraction of urban solid waste (OFMSW) that can be obtained from waste treatment plants (the existing ones and the plants under construction) amounts to about 2 Mt/year, which corresponds to about 400,000 t/year of dry matter. This value may also quadruple if plants treating all national wastes were developed.

Tab 2.5 Italian biomass residues availability: type of wastes and their quantity.

SECTOR	TYPE OF WASTE	QUANTITY (k.ton of dry matter per year)
AGRICOLTURE		
SOFT WHEAT	Straw	500
HARD WHEAT	Straw	1,600
BARLEY	Straw	380
OATS	Straw	120
RICE	Straw	550
MAIZE	Stalks/Cops	3,100
TOBACCO	Stalks	10
SUNFLOWER	Stalks	350
GRAPEVINE	Shoots	880
OLIVE TREE	Wood, branches, fronds	800
APPLE TREE		90
PEAR TREE	Branches	50
PEACH TREE	Branches	150
CITRUS TREE	Branches	480
ALMOND TREE	Branches	95
HAZEL TREE	Branches	85
ACTINIDIA	Shoots	25
APRICOT, CHERRY, PLUM, TREE	Branches	35
TOTAL	Straw, stalks, stems, leaves, etc.	9,300
FOREST BIOMASS		
HIGH FORESTS (broad-leaved trees	Branches, tops and small residues	1,800
COPPICE WOODLANDS (simple, co	Whole plant	4,700
TOTAL		6,500
AGRO-INDUSTRY RESIDUES		
SUGAR REFINERY	Molasses, dry pulp, sludge	1,570
TOMATOES	Peels and seeds	135
CITRUS FRUIT	Pulp and peels	210
FRESH FRUIT	Stones	35
DRIED FRUIT	Peels	135
FLOUR MILLING	Bran	185
PASTA INDUSTRY	Part breaking off	60
RICE INDUSTRY	Husk, chaff, starch, green grains, broken parts	520
OIL	Virgin residues, exhausted residues	750
WINE	Virgin pomace, exhausted pomace, grape stalks	300
TOTAL		3,900
WOOD INDUSTRY RESIDUES		
PRIMARY WOOD PROCESSING	Barks, wane, etc.	2,500
SECONDARY WOOD PROCESSING	Sawdust, woodchips, etc.	1,700
PAPER INDUSTRY	Pulp-paper, pulper	300
TOTAL		4,500

Nevertheless, availability of the waste materials and their cost are just two of the main criteria for the selection of feedstocks to be used in biohydrogen production.

The others are waste biodegradability, low concentration of inhibitory to microbiological activity compounds, and especially their carbohydrate content, as stated previously. For example, wastewaters from food-processing industries and breweries, and

agricultural wastewaters from animal confinements are ideal candidates for bioprocessing because they contain very high levels of easily degradable organic material, which results in a net positive energy or economic balance, even when heating of the liquid is required (Angenent *et al.*, 2004). In addition, they have typically a high water content, which circumvents the necessity to add water for bioprocessing them in wet technologies.

Considering the plant-based raw waste materials, the following main categories can be identified (Boeriu *et al.*, 2005):
- Agricultural residues from primary agricultural production, such as straw, bran, corn cobs, corn stover, foliage, and hulls;
- Agro-industrial wastes generated in the food production at various links in the chain, as well as resources lost in inefficiency of food uptake and animal production, vegetable, fruit garden-waste and compost;
- forestry residues and grass.

The majority of them are mainly lignocellulosic materials (sugar cane and sweet sorghum bagasse, corn stalks and stover, fodder maize, wheat straw, etc.) that are either poorly valorized or left to decay on the land and that are attracting increasing attention as an abundantly available and cheap renewable feedstock utilized via bioconversions (Soetaert and Vandamme, 2005). Ntaikou *et al.* (2010a) state that around 2.9×10^3 million tons from cereal crops, 1.6×10^2 million tons from pulse crops, 1.4×10 million tons from oil seed crops and 5.4×10^2 million tons from plantation crops are produced annually worldwide. In Table 2.6 different types of lignocellulosic residues used as feedstocks for hydrogen production are reported, along with their biohydrogen yields and production rates and the pretreatment adopted for their solubilization.

Tab. 2.6 Hydrogen production via dark fermentation with different types of lignocellulosic residues (Courtesy of Ntaikou *et al.*, 2010a).

Lignocellulosic residue	Pretreatment	Microorganism	Operation mode	H_2 production rate	Maximum H_2 yield (mol/mol cons. hexose)
Wood fibers	Mechanical	*Clostridium thermocellum*	Batch	–	1.47
Corn stover	Steam explosion (90–220°C, 3–5 min)	Mixed mesophilic cultures	Continuous	10.56 mmol/h	3
Sugarcane bagasse hydrolysate	Acid-thermal hydrolysis H_2SO_4 0.27–7(v/v), +121°C, 60 min	*Clostridium butyricum*	Batch	1.611 l/l/day	1.73[b]
Fobber maize juice	Mechanical	Mixed mesophilic cultures	Continuous	–	69.4[c]
Sweet sorghum residues	Mechanical	*Rumicococcus albus*	Batch	–	2.59
Wheat straw	Mechanical	*Caldicellulosiruptor saccharolyticus*	Batch	–	3.8 (44.7 l/kg dry biomass)
Maize leaves	Mechanical	*Caldicellulosiruptor saccharolyticus*	Batch	–	3.6 (81.5 l/kg dry biomass)
Barley straw	Mild acid 1.8% H_2SO_4 w/w	*Caldicellulosiruptor saccharolyticus*	Batch	–	–
Corn stalks	Mild acid 1.8% H_2SO_4 w/w	*Caldicellulosiruptor saccharolyticus*	Batch	–	–
Bagasse	Alkali-thermal 0.2–4 g/l NaOH, 100°C, 2 h	Mixed thermophilic cultures	Batch	0.28 mmol/h/g TVS	13.39[d]
Corn stover	Acid-thermal hydrolysis H_2SO_4 0.25–4(v/v), +121°C, 30–180 min	*Thermoanaerobacterium thermosaccharolyticum*	Batch	3.305 l/day	2.24

[a] l/kg TVS
[b] mol/mol total sugar
[c] ml H_2/g dry mass
[d] mmol H_2/g TVS

The second most abundant group of plant-based residues is that of agricultural or industrial wastes containing starch and cellulose. These feedstocks are easy to be used by AD processes, but have different characteristics and properties influencing the process parameter optimization. Starch containing solid wastes are generally easier to process for hydrogen gas formation because starch can be hydrolyzed to glucose and maltose by acid or enzymatic hydrolysis followed by conversion of carbohydrates to organic acids and then to H_2. Many authors studied the suitability of starch-based residues for hydrogen-production: lactate-containing wastewater, cow dung slurry, vegetable starch, sugar-cane juice and whey, bean-product wastewater, tofu wastewater have been extensively used for

biohydrogen production in lab-scale (Nath and Das, 2003). Claassen *et al.* (2005) reported the use of potato steam peels, in form of a highly viscose starch-rich slurry, obtained as a by-product in the potato processing industry and commonly used in the fodder industry (wet feed component).

One of the highest specific hydrogen production rate was 237 L_{H2}/kg_{VSS} d when edible corn starch was used as the substrate by *C. pasteurianum* (Liu and Shen, 2004), while specific yield of 480 L_{H2}/kg_{VSS} with 4.6 g/L starch concentration at 37 °C using a mixed sludge was obtained by Zhang *et al.*, 2003. Yokoi *et al.* (2001) used dried sweet potato starch residue (2.0% starch residue content) to feed a mixed culture of *C. butyricum* and *E. aerogenes*: H_2 yield obtained in long term repeated batch operations was 2.4 $mol_{H2}/mol_{glucose}$.

Differently, cellulose containing wastes (paper wastes, agricultural wastes - wheat straw, corn stover, rice straw, corn cobs...- and others) require further pre-treatment and therefore are less favorable to be used. Cellulose (and hemicellulose) content of wastes can be hydrolyzed to carbohydrates and then further processed to hydrogen, but very often these wastes must be preventively grounded and then delignified by mechanical or chemical means before fermentation.

Enlarging our interest to other wastes than those strictly plant-based, heterogeneous and complex solid wastes or wastewaters have already been tested as feedstocks for fermentative hydrogen production even if, differently from the wastes previously described, they own quite high content of proteins and fats together with carbohydrates. The different origin and composition of these wastes bring to general lower conversion (to hydrogen) efficiencies than pure carbohydrates and Lay *et al.* (2003) stated that the hydrogen production potential of carbohydrate-based wastes may be 20 times higher than that of fat-based and protein-based waste. This is possibly explained by the consumption of hydrogen towards

ammonium using nitrogen generated from protein biodegradation. Table 2.7 shows sources of possible degradable waste streams which are presently used for methane production and that, with an appropriate setting of the operating parameters, can be exploited also for hydrogen production. The waste streams are divided into solid wastes, waste slurries and wastewaters.

Tab. 2.7 Origin of organic waste streams that can be utilized for the production of biogas (adapted from Weiland, 2000).

Solid wastes	Domestic	Separately collected vegetable, fruti and yard waste
		The organic fraction of source-sorted household waste
		Organic residual fraction after mechanical separation of integral collected household waste
	Agricultural	Crop residues
		Undiluted manure
Waste slurries	Domestic	Primary and secondary sewage sludge
	Agricultural	Liquid manure
	Industrial	Slaughterhouse and meat-processing
		Fish-processing
Wastewater	Domestic	Sewage, Black
		Water sewage
	Industrial	Dairy, sugar, starch, coffe processing, breweries and beverages, distilleries and fermentation, chemical, pulp and paper, fruit and vegetable processing

These waste streams may require particular bio-processing technologies to hydrolyze their carbohydrate fraction, to remove undesirable components and for nutritional balancing. As an example, hydrogen yields of 1.2 mg_{H2}/g_{COD} were reported by Wang *et al.* (2003a) when waste sludge (biosolids) generated in wastewater treatment plants was used as the raw material, with higher yields (15 mg_{H2}/g_{COD}) obtained from the sludge filtrate.

Moreover they may show different conditions for optimal fermentation: for example, cheese whey, a rich in readily fermentable sugars wastewater, reached its highest hydrogen yield

and production rate both at pH between 6 and 7 (Davila-Vazquez *et al.*, 2008) and at pH in the range 4 - 5 (Yang *et al.*, 2007).

However, among all wastes, the research activity on fermentative H_2 production mainly focuses on food-related wastes, e.g. food-industry wastes/wastewaters or municipal solid organic wastes. Even if these show high variations in carbohydrate and protein types and concentrations in the mixture, they are commonly high-strength organic wastes, whose biotransformation to biohydrogen can be considered particularly appealing from both the environmental and the economic standpoint (Ntaikou *et al.*, 2010a). Rice winery, noodle, sugar, and molasses manufacturing, olive mill wastewater, olive pulp, dairy industry, baker's yeast, brewery wastewaters and cheese whey were successfully tested for hydrogen production at laboratory scale (Table 2.8; Courtesy of Ntaikou *et al.*, 2010a).

Tab. 2.8 Hydrogen production via dark fermentation from different types of waste and wastewaters (Courtesy of Ntaikou *et al.*, 2010a).

Type of waste/ wastewater	Microorganism	Operation mode	H_2 production rate	Maximum H_2 yield
Sugar factory wastewater	Mixed thermophilic culture	Continuous	4.4 l/l/day	2.6 mol/mol hexose
OFMSW	Mixed mesophilic culture	Batch	0.4 l/g VSS/day	0.15 l/g OFMSW
Rice winery wastewater	Mixed culture	Continuous	9.33 l/g VSS/day 3.81 l/l/day	2.14 mol/mol hexose
Food waste—sewage sludge	Mixed mesophilic culture	Batch	2.67 l/g VSS/day	122.9 ml/g COD carbohydrate
Food waste	Mixed thermophilic culture	Batch	0.288 l/g VSS/day	1.8 mol/mol hexose
Cheese whey	*Clostridium saccharoperbutylacetonicum*	Batch	28.3 ml/h	7.89 mmol/g lactose
Potato processing wastewater	Mixed mesophilic culture	Batch	–	2.8 l/l wastewater
Cheese whey	Mixed mesophilic culture	Batch	–	10 mM/g COD
Dairy wastewater	Mixed mesophilic culture	Continuous	1.59 mmol H_2/l/day	–
Molasses	Mixed mesophilic culture	Continuous	4.8 l/l/day	–
Cheese whey	Mixed mesophilic culture	Batch	8.1 mmol/l/h	5.9 mol/mol lactose
Cheese whey	Mixed mesophilic indigenous microbial culture	Continuous	2.51 l/l/day	0.9 mol/mol hexose
Olive pulp	Mixed mesophilic culture	Continuous	0.26 l/l/day	0.19 mol/kg TS
Olive oil mill wastewater	Mixed mesophilic culture	Continuous	201.6 ml/day	196.2 ml/g hexose
Wastepaper	*Ruminococcus albus*	Batch	–	2.29 mol/mol hexose (282.76 l/kg dry biomass)

The hydrogen yield obtained from food wastes/wastewaters range from 0.7 and 2.7 mol_{H2}/mol_{hexose}, results quite comparable to those

from pure carbohydrates. An interesting study was made by Van Ginkel *et al.* (2005a), who reported hydrogen production from four different food-processing industries (two confectioners and apple and potato processing industries). The H_2 production rates obtained were in the range of 0.1 and 2.8 $L_{H2}/L_{wastewater}$, with potato wastewater as the best performing waste.

Food wastes were also used in different studies aiming at optimizing some of the factors regulating the process. Shin *et al.* (2004) reported higher H_2 production potential and production rates from food wastes under thermophilic conditions than in mesophilic processes. The effect of HRT on H_2 production from food wastes was studied by Han and Shin (2004a), who obtained 58% COD reduction and 70% hydrogen formation efficiency at very fast retention time of 5.3 h.

Market bio-wastes were also used in our works individually (Paper II), showing promising biohydrogen production potential of 176 ± 2 NL_{H2}/kg_{VS}, or co-digested with swine slurry (Paper I and III). In particular market bio-wastes/swine manure ratio of 35/65 and HRT of 2 d gave the highest production rate (among the tested conditions) of 3.27 ± 0.51 $L_{H2}/L_{reactor}$ d, with a corresponding hydrogen yield of 126 ± 22 $NL_{H2}/kg_{VS\ added}$ and H_2 content in the biogas of 42 ± 5%. At these operating conditions the process exhibited also one of the highest measured stability, with daily productions deviating for less than 14% from the average (Paper I).

Municipal solid waste (MSW) is generally defined as "household waste plus other waste of a similar composition collected by (or on behalf of) the local authority". In practice, this means that if the waste generated by a particular commercial business is collected along the household waste the material is classed as MSW. The combustion of MSW for energy production is an effective use of wastes that significantly reduces the problems of waste disposal but it generates greenhouse gas emissions. Alternatively, a fraction of municipal waste can be composted, although its main part is

typically landfilled, which is being severely curtailed due to unavailability of land and environmental concerns. So, AD processes seem to be a valid and environmental-friendly alternative.

The biological hydrogen production from organic fraction of municipal solid wastes (OFMSW) is quite promising, since this waste can represent up to 70% of the total MSW produced, consisting of paper (up to 40%), garden residues, food wastes and wood. With specific regards to Italy, in 2008 it was reported that the total municipal solid waste (MSW) production in Italy is about 2 Mt/year (ITABIA, 2008). The process feasibility was investigated by many authors, such as Lay *et al.* (1999) who used different mixed anaerobic microflora under mesophilic conditions for OFMSW digestion. Varying the "food-to-microorganisms" ratio they reported that at 0.4 $g_{OFMSW}/g_{bacterial\ biomass}$, high hydrogenic activity took place, with 43 L_{H2}/kg_{VSS} h specific production rate and 125 L_{H2}/kg_{VS} h production potential. Okamoto *et al.* (2000) reported a hydrogen production of 19.3-96.0 $L_{H2}/kg_{VSadded}$ by mesophilic batch fermentation of MSW composed mainly by rice and carrots. Similarly, Valdez-Vazquez *et al.* (2005) reported a yield of 95 $L_{H2}/kg_{VSadded}$ with semi-continuous CSTR treating municipal organic wastes.

In our research (Paper II), the biohydrogen potential (BHP) test applied to a representative sample of OFMSW collected in northern Italy achieved very high hydrogen yield (202 ± 3 $L_{H2}/kg_{VSadded}$) in thermophilic condition. Considering also the huge amount available of this waste, OFMSW could represent a huge source of renewable energy if AD and hydrogen economy would take place in our Country. To gain an insight into possible production rates of real scale (CSTR) plant, typically digesting highly concentrated organic mixtures (50–150 g_{VS}/L), a concentrated organic mixture of OFMSW (126 g_{TS}/L) was also tested in Paper II through a continuous lab-scale fermenter. Hydrogen production (60 ± 4 $NL_{H2}/kg_{VS\ added}$), although

comparable with previously mentioned results, resulted in only 30% of its BHP, showing that further improvements are still needed for future full-scale applications of dark fermentation.

Chapter 3

Biohydrogen production via bioelectrochemical system: the MEC application

3.1 General operating principles

Bioelectrochemical systems (BES) use the catabolism of living microbial cells to convert reducible organic materials to:
- electricity, in microbial fuel cells (MFCs);
- hydrogen, in microbial electrolysis cells (MEC);
- other products (Rabaey and Rozendal, 2010).

These systems, based on the bacteria ability to generate electric potential and firstly exploited in MFCs, result nowadays in a number of different applications and could be overall named "MxCs" (Logan, 2010). For example, it is possible to include those systems that, through the addition of external voltage to the potential generated by

the bacteria, generate other products than hydrogen, such as methane and hydrogen peroxide (Cheng *et al.*, 2009; Liu *et al.*, 2005; Rozendal *et al.*, 2006a) and those MFCs that through a specific technique use membranes to allow for simultaneous water desalination and electrical power production (Cao *et al.*, 2009; Kim and Logan, 2011).

The first connection between electricity and biology was made in 1791 by Luigi Galvani, who discovered that severed frog's legs contract every time the muscle and nerve endings are connected to a static electricity generator (Wrana *et al.*, 2010). More than a century spent since, in 1911, M.C. Potter discovered the ability of certain bacteria to produce electrical current (Potter, 1911): however no practical applications of this ability was firstly assumed. Then followed experiments about bacteria-electrode interaction (by examining overall potentiometric intensities of chemical reactions during bacterial growth; Cohen, 1931) and, during the '50s and '60s, about converting organic material into electrical energy. For real, it was just with the '70s, under the boost of US space program which saw MFCs as potential waste disposal units that could generate power during space missions (Shukla *et al.*, 2004), that this new biological technology began to be explored. During those years it was already well known that when bacteria oxidize a chemical, electrons are captured and transferred to a series of respiratory enzymes used to store energy (in the form of ATP) within the cell. These electrons are then released to an electron acceptor such as iron, nitrate, sulfate, or oxygen. This biochemical property let the scientific community to assume that the same bacteria that can respire using iron could be able to transfer electrons to an insoluble acceptor, such as an electrode, acting as a catalyst for oxidizing the organic matter (Shukla *et al.*, 2004). However, despite an improved understanding of the biological mechanisms, the laboratory experiments of the 70's showed several discouraging results:

maximum power densities reached were low, chemical mediators used to ease the transfer of the electrons from the microorganisms to the electrodes had to be often added into the medium and were toxic, and rich media were needed to cultivate the bacteria (Allen, 1972). Essentially, it was determined that current could not be produced at a rate or quantities large enough to be a viable source of electrical energy (Wrana *et al.*, 2010) and the research on this field was (temporarily) stopped.

Finally, several events happened at the same time that completely changed the prospects for electrical current generation by microbial fuel cells (MFCs). It was discovered that:

- mediators did not need to be added into solution as bacteria capable of direct extracellular electron transfer to fuel cell anodes were found (Kim *et al.*, 1999);
- wastewaters could be used as a source of fuel, while accomplishing wastewater treatment (Liu *et al.*, 2004);
- much higher power densities were attained through cell architecture and parameters optimization (Rabaey *et al.*, 2003).

Hereinafter these features, together with the basis of the BES technologies, will be discussed.

A MFC acts very similarly to a conventional fuel cell, where hydrogen gas is injected into the anode chamber and usually is split on a platinum-coated electrode into protons and electrons (see chapter 1). In a MFC organic matter is used instead of hydrogen and its breakdown into protons and electrons (and CO_2) is accomplished by electrochemically active microbes growing on the surface of the anode. From here the electrons and protons travel through an external circuit and electrolyte solution, respectively: the former creates an electric current, while the latter uses oxygen as the electron acceptor at the (usually Platinum-coated-) cathode generating just water as reaction product (Figure 3.1).

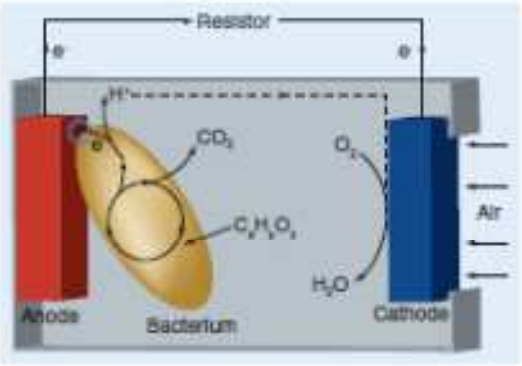

Fig. 3.1 Single chamber air cathode microbial fuel cell showing the anode, where bacteria form a biofilm on the surface, and the cathode, which is exposed to the air (Courtesy of Liu *et al.*, 2010).

Specific bacteria, naturally present in waste organic matters, are involved into the process and permit to avoid the expensive addition of chemical mediators to the culture. These microorganisms capable of exocellular electron transfer have been previously referred to as electricigens or anode-respiring bacteria and are now widely known as exoelectrogens (Liu *et al.*, 2011). Even if generally there is a predominance of the same type of microorganisms on the anode surface, the microbial community appears to be highly dependent upon the design and the operational parameters of the MFC system and even upon the culture technique (batch or flow-through mode). For example, in systems harvesting electricity under highly anoxic conditions, *Geobacter* species were found to be the predominant organisms involved in electricity production, while in other systems, where the reactor design permits substantial leakage of oxygen into the anode chamber, organisms more tolerant to oxygen exposure could predominate (Lovley, 2006; Rabaey *et al.*, 2004).
All these bacteria rely on three different strategies of extracellular transport of electrons, well described by Wrana *et al.* (2010) (Figure 3.2):

a. Direct electron transfer using outer membrane c-type cytochromes;
b. Long range electron transfer via biogenic soluble mediators (shuttle);
c. Long range electron transfer via conductive bacterial appendages (conductive pili or microbial nanowires).

a. Microbial strains with mutations or deletions in some genes that encode for outer membrane cytochromes and for direct and indirect mineral reduction (cyma and omcB for *S. oneidensis*; omcS and omcE for *G. sulfurreducens*) gave evidence of the role of these c-type cytochromes in direct electrical contacts between microbe and electrode (Holmes *et al.*, 2006; Lies *et al.*, 2005).

b. Certain microorganisms produce soluble exogenous mediators that shuttle electrons from cells to insoluble compounds via diffusion (Wrana *et al.*, 2010). Specifically, riboflavin secretion for this transfer has been observed both in *Shewanella sp.* and *Geothrix fermentans*. The main disadvantage of this mechanism is that it is energetically taxing and may not be the most desirable system for the bacteria (Mahadevan *et al.*, 2006).

c. Unlike other pili that aid bacteria in cell motility or adhesion to solid surfaces, nanowires are electrically conductive protein filaments (composed of the repeated single unit PilA; Reguera *et al.*, 2005), that enable communications between microorganisms. Therefore they are responsible for maximizing biofilm health by coordinating a cooperative electronic community and by aggregating and interconnecting cells into a network capable of effectively distributing and dissipating electrons (Wrana *et al.*, 2010; Figure 3.2). In MFCs they enable communications both between the microorganisms composing the biofilm and between the biofilm and the electrode.

Among the mechanisms described, one may be dominant in the electron transfer, but it is known the possibility of the involvement of all three mechanisms in electron transfer by a single bacterial specie.

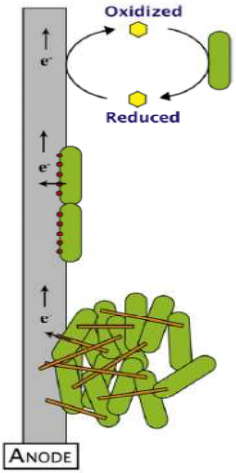

Fig. 3.2 Mechanisms for electron transfer from the exoelectrogenes to the anode: i) Long-range electron transfer via electron shuttles (yellow hexagon), ii) direct electron transfer via outer-surface c-type cytochromes (red circles), and iii) long-range electron transfer via conductive pili or "microbial nanowires" (orange rods). (Courtesy of Wrana *et al.*, 2010).

The substrate exploitable by MFC is another advantage of this technology, as current can be produced by using various organic matters, from the simpler ones, such as acetate, lactate, and glucose, to more complex materials, such as domestic and industrial wastewaters. In 2004, for the first time microbial fuel cells were successfully applied to real (domestic) wastewaters treatment, simultaneously generating electricity (26 mW/m^2) and removing up to 80% of the BOD win the wastewater (Liu *et al.*, 2004). In the following years many other wastewaters have been successfully exploited through MFC technology (Table 3.1, Courtesy of Logan, 2005).

Tab. 3.1 Real complex wastewaters used in MFC and the achieved power density (Courtesy of Logan, 2005).

Substrate	Power (mW/m^2)
Starch wastewater	19
	20
Anaerobic sediments	16
	28
Domestic wastewater	
Continuous – large SCMFC	26
Flat plate	76
Batch, small SCMFC (PEM)	28
Batch, small SCMFC (no PEM)	146
Animal wastewater	260

Lastly, just in recent years, microbial fuel cells with an enhanced power output have been developed, providing possible opportunities for practical applications (Rabaey and Verstraete, 2005). Since 1999, power production has increased by five to six orders-of-magnitude (based on projected surface area; Logan, 2010). Power densities of MFCs using oxygen have reached from 2.7 W/m^2 (power normalized to the cathode; Xing *et al.*, 2008) up to 6.9 W/m^2 (much larger cathodes than anodes, power normalized to the anode area; Fan *et al.*, 2008). However, in flow-through systems required for treating large volumes of liquid waste, the highest power outputs are less than 2 $W/m^2_{\text{anode surface}}$, even when treating readily degradable pure substrates like glucose (Lovley, 2006). This power output is unlikely to be sufficient to recover the power expended in pumping the fluid through the system. Therefore, scaling these laboratory systems to the size that would be required to handle large volumes of wastes is still an issue.

These preliminary remarks are useful to better understand MEC (microbial electrolysis cell) technology, which represents the next generation of the MFCs, converted into an innovative way for biological hydrogen production. They also share some operational

and functional advantages of MFCs, such as direct and high efficient conversion of organic substrate energy into usable energy (H_2) or efficient operation at ambient, and even at low, temperatures (differently from all the current bio-energy processes).

MECs were independently realized by two research groups (Liu and Logan, 2005; Rozendal and Buisman, 2005) just a few years ago and firstly named as BEAMR (bio-electrochemically assisted microbial reactor) or BEC (biocatalyzed electrolysis cells) (Liu *et al.*, 2010). MECs share many attributes with MFCs because the design of the anodes and the electrogenic reactions occurring are similar. However, differently from a MFC, in a MEC the protons generated on the anode surface by biomass breakdown by microbes combine between themselves and the electrons released at the cathode to generate hydrogen (Figure 3.3).

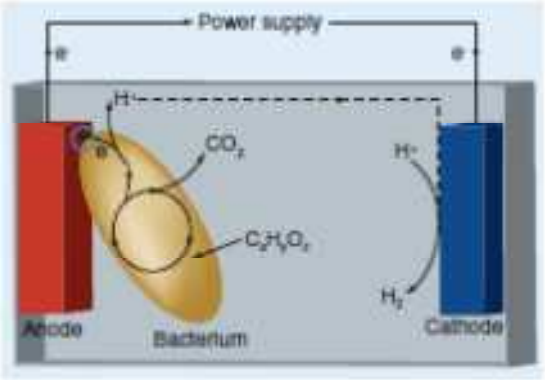

Fig. 3.3 Single-chamber microbial electrolysis cell with a power supply as the driving force for electron flow from anode to cathode (Courtesy of Liu *et al.*, 2010).

Another operational difference is that reaction at the cathode must occur in an anaerobic environment. This simplifies cathode design, but, since the product is a gas rather than electricity, the cell architecture must be modified for collecting this gas (see details in section 3.2) (Logan *et al.*, 2008). Moreover, while in MFC oxygen diffusion into the anode chamber substantially reduces recovery of

electrons from substrate as current (defined as Coulombic Efficiency, CE), in MEC the lack of oxygen results, on average, in greater CE. A fully anoxic MEC also enables a better growth of strict anaerobes, but simultaneously the lack of exposure of the microorganisms to oxygen enhances the likelihood for methanogenesis, which can lower hydrogen recovery (Call and Logan, 2008; Rozendal *et al.*, 2006a).

More important, if in MFC, due to the higher redox potential of oxygen than that of the microbial anode, electrons flow spontaneously from the anode to the cathode generating electricity, in MEC the reduction reaction of H^+ ions to H_2 at the cathode has a lower redox potential than the anode, thus the electrons do not flow spontaneously through the circuit and the hydrogen gas generation is not spontaneous. Therefore MECs require the addition of energy to overcome the thermodynamic limitations set by the chemical reactions at the electrodes and by the potential losses occurring within the system.

In details, under standard biological conditions (T = 25 °C, P = 1 bar, pH = 7) the Gibbs free energy of reaction ($\Delta G_r^{0'}$) for acetate oxidation to hydrogen is positive and therefore acetate cannot be fermented to hydrogen:

$$CH_3COO^- + 4\ H_2O \rightarrow 2\ HCO_3^- + H^+ + 4\ H_2 \quad (\Delta G_r^{0'} = +104.6\ kJ/mol)$$

Additional energy added to the system in order to favor hydrogen evolution is supplied by applying a small voltage, named applied voltage (E_{ap}). In order to drive the H_2 production, the applied voltage needs to be at least larger than a value, usually referred as the equilibrium voltage (E_{eq}), which could be calculated as:

$$E_{eq} = -\frac{\Delta G_r^{0'}}{nF}$$

where n is the amount of electrons involved in the reaction, and F = 96 485 C/mol e^- is Faraday's constant.

Alternatively it could also be calculated as the theoretical overall cell potential expressed as an electromotive force (E_{emf}), by evaluating the difference between the cathode and the anode theoretical potentials ($E_{electrode}$):

$E_{emf} = E_{Cat} - E_{An}$

Each $E_{electrode}$ can be calculated from tabulated values under standard conditions by using the Nernst equation, which is:

$$E_{electrode} = E^{0}_{electrode} - \frac{RT}{nF} \ln \frac{[reduction]^{p}}{[oxidation]^{r}}$$

where R is the universal gas constant (8.31447 J/mol K) and T is the absolute temperature.

Following the example of acetate oxidation to H_2 and considering the following two half-reactions occurring at the anode and at the cathode (under standard biological conditions),

ANODE: $CH_3COO^- + 4\ H_2O \rightarrow 2\ HCO_3^- + 9\ H^+ + 8\ e^-$;
CATHODE: $2\ H^+ + 2\ e^- \rightarrow H_2$,

Eeq is very low:

$$E_{eqACETATE} = -\frac{104.6 \times 10^3}{8 \times 96485} = (-0.414\ V) - (-0.279\ V) = -0.14\ V$$

The negative sign indicates that the reaction is not spontaneous and that a voltage has to be applied in order for the reaction to proceed; also, this value translates to a theoretical energy requirement of 0.29 $kWh/m^3\ H_2$.

For real, depending on the substrate consumed at the anode by the bacteria and on the operating conditions, including hydrogen partial pressure, pH, resistance in the system and polarization at the electrodes, the *Eeq* could vary.

Also, under typical operating conditions, the applied voltage (*Eap*) is always larger than *Eeq* because of internal losses in the system. These losses (anodic and cathodic overpotential and ohmic losses)

are all a function of the current, are comparable to those observed for MFCs and they will be explained in details in section 3.2. Experiments have shown that the microbial electrolysis reactions typically start to occur at *Eap* above 0.2 V, which corresponds to an energy requirement of 0.43 kWh/m^3 H_2 (at 100% cathodic hydrogen recovery; Logan *et al.*, 2008). For real, even applied voltages lower than 0.3 V may result in a low hydrogen-production rate and erratic system performance, while E_{ap} > 1 V are not recommended because the electrical energy input would be equal or larger than that of the water electrolysis process (1.2 V; Liu *et al.*, 2010).
E_{ap} is typically provided by a power supply unit or a potentiostat, two different external power source devices that are able to provide the low voltages required by MEC (Logan *et al.*, 2008).

Evaluating the performance of this novel technology, recently the overall MEC efficiencies and hydrogen production rates have increased. Cheng and Logan (2007) reported that hydrogen production yield can reach high value of 2.0 - 3.95 $mol_{H2}/mol_{acetate}$ (representing the 50-99% of the theoretical maximum) by improving the materials and reactor architecture in a single chamber MEC. Also, increasing the applied voltages from 0.2 to 0.8 V, the production rate increased from 0.03 to 1.5 $m^3_{H2}/m^3_{reactor\ (total\ volume)}$ d, simultaneously reducing the time needed for a complete batch cycle (from 30 h to 3 h). At the same time the energy efficiency (η) of the system ranged from η_W = 681-243% (when evaluated in terms of only the voltage addition) to η_{W+S} = 62-86% (when evaluated on the basis of both the voltage added and the heat of combustion of the acetate added). The biogas produced was nearly pure hydrogen (≈ 99.5%) with only trace amounts of CO_2 and CH_4 in all experiments.
The same authors reported hydrogen production in MEC at high yields from a variety of substrates (Table 3.2; Courtesy of Cheng and Logan, 2007). Glucose was converted to hydrogen gas at a rate (1.23 m^3 H_2/ $m^3_{reactor\ (total\ volume)}$ d) similar to that of acetate but at a lower

overall maximum recovery of 71% (8.55 $mol_{H2}/mol_{substrate}$). This recovery is 4- to 5-times larger, however, than that typically achieved through cellulose fermentation (Cheng and Logan, 2007). Even all the predominant acids typically produced by glucose fermentation (acetic, butyric, lactic, propionic, and valeric acid) were successfully used to generate H_2 in the electrohydrogenic process at energy recoveries (η_{W+S}) of 66-82% (Table 3.2).

Tab. 3.2 Hydrogen production using cellulose, glucose, or five different organic acids at an applied voltage of 0.6V (Courtesy of Cheng and Logan, 2007).

Substrate	Y_{H_2}, mol of H_2/mol of substrate	R_{H_2}, %	Production rate, $m^3 \cdot d^{-1} \cdot m^{-3}$	η, %	η_{W+S}, %
Glucose	8.55	71	1.23	266	64
Cellulose	8.20*	68	0.11	268	63
Acetic acid	3.65	91	1.10	261	82
Butyric acid	8.01	80	0.45	285	77
Lactic acid	5.45	91	1.04	283	82
Propionic acid	6.25	89	0.72	248	79
Valeric acid	8.77	67	0.14	263	66

*Calculated per mole of hexose equivalent.

Improving the single-chamber membraneless MEC architecture, by using graphite fiber brush anode and acetate as feeding, in 2008 Call and Logan achieved a very high cathodic hydrogen recovery (78-96%) and production rate (3.12 ± 0.02 m^3 H_2/ $m^3_{reactor\ (total\ volume)}$ d). This production rate is more than double than that obtained in previous MEC studies and demonstrates how fast this technology is improving towards higher production rate (which has increased more than 100-fold in less than 5 years) and higher recover efficiency. An updated overview of the major MEC systems reported in literature has been summarized together with their performance and the adopted parameters (applied voltage, cathode type, membrane, substrate...) by Liu *et al.* (2010) (Table 3.3).

As Table 3.3 shows, the current density generated by the single-chamber MECs (4.2-12 A/m^2) is generally higher than that by the two-chambers MECs with membranes (0.4-3.3 A/m^2). The highest value of 12 A/m^2 has been achieved in a single-chamber MEC with

equal-sized anode and cathode and it is almost the half of the highest microbial anodic current density (26 A/m^2) obtained in MFC with carbon cloth anode and a large air cathode (Fan *et al.*, 2008). The same Table shows also that MECs energy efficiencies based on electricity input (η_W) range typically between 114% and 270%, and they are expected to increase through further reduction of internal losses.

Thus, big efforts are needed in improving the architectural MEC designs and the materials used in the process (aiming at increasing the electrode-area-to-volume ratio) and in avoiding those factors potentially limiting H_2 production (such as hydrogen consumption by hydrogenotrophic methanogens or the direct acceptance of electrons from the cathode by electromethanogenic bacteria). Indeed, although exoelectrogens generally can outcompete methanogenic bacteria for acetate on a MEC anode, a significant amount of methane can be detected in single-chamber MECs after a few weeks operation, especially with the use of wastewaters as substrate (Call and Logan, 2008; Hu *et al.*, 2008). Also in our work (Paper IV), where single chamber MECs with different cathodes (stainless steel, MoS_2 and Platinum) were fed with industrial wastewater with high-content of methanol, persistent and high methanogenesis (always > 55%) was detected. This persuaded us to compare MEC with (simulated) anaerobic digestion process.

In the next section (3.2) some of the main MEC regulating factors will be presented, together with recent studies aiming at their optimization.

Tab. 3.3 Components and performances of microbial electrolysis cell system by microbial cell design (Courtesy of Liu *et al.*, 2010).

System description				System performances							Ref.
Anode	Cathode/Pt mg/cm²	Membrane	Substrate	E_{ap} (V)	Liquid volume (ml)	I_A[1] (A/m²)	C_E[2] (%)	r_{H2}[3] (%)	n_w[4] (%)	Q_{H2}[5] m³/d/m³	
Two-chamber system											
Carbon cloth	Carbon paper/0.5	Nafion*	Acetate	0.6	200		78	72			[4]
Graphite felt	TI mesh/0.5	Nafion	Acetate	0.5	6600	0.5		53		0.02	[6]
	TI mesh/0.5	CEM	Acetate	1	3300	2.4	23	23	148	0.33	[40]
	Graphite felt, biocathode	CEM	Acetate	-0.7*	250	1.2		<49[6]		0.63	[58]
		CEM	Acetate	-0.7*	250	3.3		<21[6]		0.04	[55]
	TI plate/0.5	Nafion	Acetate	0.8	360	2.8	62	53		0.052	[65]
Graphite granules	Carbon cloth/0.5	AEM	Cellulose	0.6	42			68	268	0.11	[7]
		AEM	Acetate	0.6	42		96	91	261	1.1	[7]
Carbon paper	Carbon paper/0.5	Nafion	Domestic WW	0.5	512	0.2	23	9.9			[5]
	Carbon paper/2	PEM	Acetate	0.35	900	0.4	33	32		0.015	[66]
Carbon felt	Carbon cloth/0.5		Acetate	1	50	4.7	95	97.5		6.32	[41]
Single-chamber system											
Carbon cloth	Carbon cloth/0.5		Acetate	0.6	300	9.3	75	63	204	0.53	[38]
	Carbon cloth + NiMo		Acetate	0.6	18	12	75	65	182	2.0	[56]
	Carbon cloth + NiW		Acetate	0.6	18	9	73	55	114	1.5	[56]
Graphite brush	Stainless steel brush		Acetate	0.6	28	88 A/m²[8]		<83[6]	221	1.7	[54]
	Stainless steel + NiO_x		Acetate	0.6	28	131 A/m²[8]	108	56	137	0.76	[57]
	Carbon cloth/0.5		Acetate	0.6	28	7.4	96	92	254	1.99	[37]
	Carbon cloth/0.5		Acetate	0.8	28	11.6	98	94	194	3.12	[37]
	Carbon cloth/0.5		Swine WW	0.5	28	4.2	29	17	190	0.9	[94]
	Carbon cloth/0.5		Glycerol	0.9	28	8.8	104	82	139	2.0	[74]
	Carbon cloth/0.5		Cellulose	0.5	28		73	63	270	1.11	[85]
	Carbon cloth/0.5		Fermentation effluent	0.6	26	5.6	87	83	252	1.41	[75]
Graphite granules	Carbon felt		acetate	1.06	140	50 A/m²[8]	60	59		0.57	[67]

*Poised cathodic potential vs Ag/AgCl.
[1]Cathodic hydrogen recovery, the percentage of current used in producing hydrogen on the cathode.
[2]Volumetric current density, calculated based on solution volume.
[3]I_A: Current density, calculated based on cathode or anode projected surface area; [4]C_E: Coulombic efficiency, the percentage of substrate used in producing current; [5]r_{H2}: Hydrogen recovery, the percentage of substrate used in producing hydrogen on the cathode; [6]n_w: Energy efficiency, the ratio of energy recovered as hydrogen over electrical energy input; [7]Q_{H2}: Hydrogen production rate, calculated based on liquid volume.
AEM: Anion-exchange membrane; CEM: Cellulose ester membrane; PEM: Proton-exchange membrane; WW: Wastewater.

3.2 Main process regulating factors and their optimization

- MEC architecture and its influence on performance

MEC could have several different architectures, but typically they are a two-chamber system with the anode chamber separated from the cathode chamber by a membrane. Figure 3.4 shows how many different designs, used for batch or continuous tests, have been

developed in the past few years. The cells can be bottle-type, cube-type, disc-type or rectangular-type, with both the electrodes soaked in solution and collection of the hydrogen produced in the headspace of the cathode chamber.

Model A and E (Figure 3.4) are the first systems designed and thus are not optimized for performance: in particular the H-type reactor (A) developed by Liu *et al.* (2005) had a high internal resistance caused by the large anode-to-cathode distance (with the electrodes placed in two distinct bottles) and by the small size of the membrane between them. However the authors reached yield of 2.2 $mol_{H2}/mol_{acetate}$ (CE = 60%; E_{ap} = 0.25 V).

Model B represents a larger reactor that increased the anode surface area using graphite granules and reduced the electrode spacing, but that didn't increase performance.

With model D (Cheng and Logan, 2007) performances were really increased, due to the adoption of a larger membrane surface, relative to the electrode-projected surface areas, to the membrane type (which allowed charge transfer via phosphate buffer anions), to the higher anode surface area (graphite granules) and to the architecture (a cube-shaped reactor with small electrode spacing). The advantages of this design include the production of relatively pure hydrogen gas in the cathode chamber, the reduction of biocontamination of cathode metal catalysts and the control of different microbial species or communities in the anode and the cathode chambers, which may be especially required for MECs with microbial biocathode (Liu *et al.*, 2010).

Coming from model D alteration, a cubic-shape MEC (model C) without a membrane, thus single-chamber type, was firstly proposed by Call and Logan (2008), in order to further simplify the reactor design and to reduce the capital costs. Removing the membrane brings some advantages that will be dealt in the "*Membranes*" section of this chapter. This cell also adopted a graphite fiber brush

anode to provide a high surface area for the exoelectrogenes, and a cathode placed in close proximity to the anode. Improved current densities were reported for this model, which also reached a maximum hydrogen production rate of 3.12 m^3_{H2}/ $m^3_{reactor\ (total\ volume)}$ d at an applied voltage of 0.8 V over a fed-batch cycle time of 12 h, together with high CEs (92% average) and cathodic hydrogen recovery between 78% and 96%. The main disadvantage of this design is the likely hydrogen consumption by methanogens growing on the cathode or in the solution, especially with actual wastewaters used as feeding. Therefore further work is needed to investigate the long-term stability of this system and to avoid methane generation.
Aiming at real scale scalability of MEC technology (no membrane, no buffer or amendments addition and wastewaters exploitation), model C was adopted in our study (Paper IV).
Other novel versions of the single-chamber MEC have been recently presented (Figure 3.5; models H, I and J), which use different materials for the main body (plastic tube, a glass bottle, a glass tube...) and aim at miniaturizing the system (Hu *et al.*, 2008; Hu *et al.*, 2009; Lee *et al.*, 2009). Although no membranes are used in single-chamber system, a separator, such as a J-cloth, is typically used to avoid a short circuit when the electrode distance is reduced to decrease the internal resistance (Liu *et al.*, 2010).

As for the models used for continuous flow tests, Rozendal *et al.* (2007) examined a MEC design using a membrane electrode assembly (MEA) architecture, i.e. a membrane integrated with the cathode and a platinum catalyst (1 g/m^2) layer faced a gas collection chamber (Figure 3.4, model F). This eliminated the liquid surrounding the cathode and reduced the reactor volume of the cell. This study achieved a hydrogen production rate of 0.3 m^3_{H2}/ $m^3_{reactor\ (total\ volume)}$ d with an E_{ap} of 1.0 V.

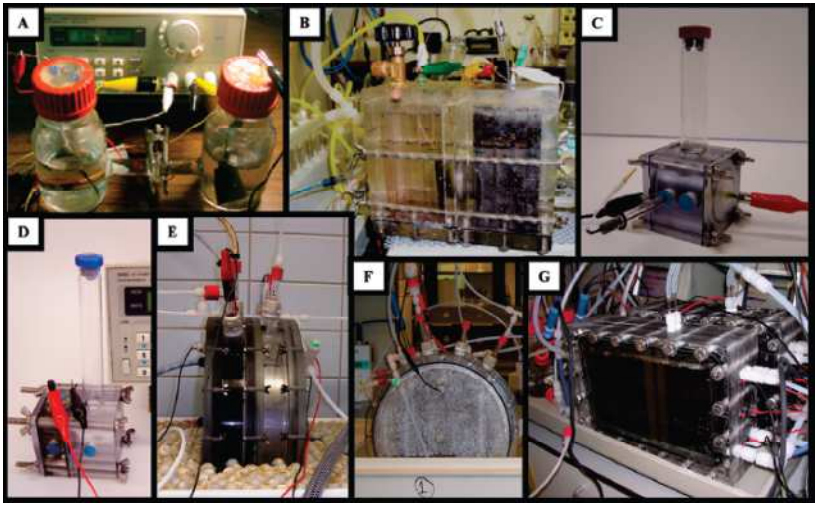

Fig. 3.4 MECs used in different studies.
Types used in fed-batch experiments: (A) H-type construction using two bottles (320 mL each) separated by a membrane; (B and D) two cube-type MECs (512 and 42 mL, respectively) where anode and cathode are separated by a membrane; (C) cube-type single chamber (28 mL) MEC lacking a membrane.
Types used in continuous flow tests: (E) Disc-shaped two-chamber MEC (each chamber 3.3 L); (F) disk-shaped membrane electrode assembly MEC (3.3 L) with gas diffusion electrode; (G) rectangular-shaped MEC with serpentine-shaped flow channels through the reactor that allow the gas to be released at the top of each flow path (each chamber 280 mL) (Courtesy of Logan *et al.*, 2008).

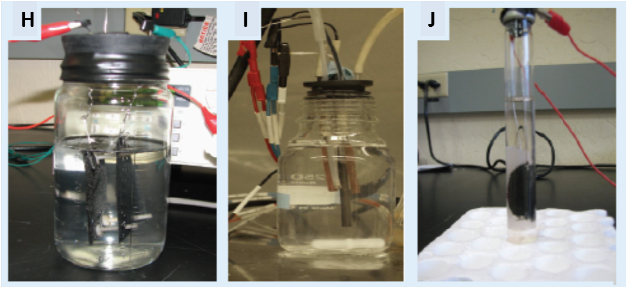

Figure 3.5 Single-chamber microbial electrolysis cell (MEC) systems: (H) Bottle MEC with carbon cloth anode and cathode; (I) Bottle-type MECs with graphite rod electrodes; (J) Tube-type MEC with carbon cloth anode and cathode separated by cloth (Courtesy of Liu *et al.*, 2010).

Another continuous vertically-orientated flow reactor architecture was recently tested, together with an innovative biocathode (Figure 3.4, model G). The cell contained many parallel flow channels and the produced hydrogen gas was continuously collected from the headspace at the top of each flow channel, while development of stagnant areas on the electrode, potentially inhibiting biocathode performance due to pH increase, was prevented.

- *Electrode materials*

Electrodes should be highly conductive (with low overpotentials) and non-corrosive, should possess a high specific surface area, should be non-fouling, inexpensive, easy to fabricate, scalable and versatile in morphologies, and should assure a good adhesion to bacteria, in order to achieve good electrical connection (Logan, 2008; Wrana *et al.*, 2010). Carbon and graphite meet many of these properties and therefore are the most used materials.

ANODE: Although carbon materials tend to corrode at high potentials on the oxygen electrode side during water electrolysis, they are chemically stable under the anaerobic anodic process in MECs (Liu *et al.*, 2010). Carbon materials with high surface area have already been used in MEC as anodes able to increase the number of bacteria attached to the surface and thereby increasing anodic current output. They include carbon cloth, carbon paper, graphite felt, graphite granules (with a graphite rod inserted into the bed of granules as a current collector), and graphite brushes (Logan *et al.*, 2008). For the brush, the graphite fibers are usually connected to a core made by twisted wires of a conductive and noncorrosive metal (such as titanium or stainless steel) while for the other shapes the electrode is pressed or glued to an insulated wire. Graphite brush and granules are widely used, especially because they maximize the surface/volume ratio and enhance the biofilm surface. However, there is also a limit to this because small pores can become clogged

rapidly by bacteria, which could die off and hence decrease the active surface of the electrode before lysis (Rabaey and Verstraete, 2005). Compared to other anode materials, these two anodes set two main challenges:

- they can add electrode resistance to the system (due to loosely packed graphite granules that may be disconnected and to the lack of contact between the fibers in the brush)
- they can bring to large average distance between the anode and the cathode: reducing this distance is the most direct way to reduce internal resistance due to low proton concentration in MEC system (Liu *et al.*, 2010).

Cheng and Logan (2007) filled the anode chamber of a two-chambers MEC with graphite granules, increasing total electrode surface area to 528 cm^2 (assuming an average particle size of 4 mm). At an applied voltage of 0.6 V and coulombic efficiency of 88%, 3.5 $mol_{H2}/mol_{acetate}$ were generated. The same authors (Cheng and Logan, 2007b) suggested also to pretreat the carbon-based anodes with a high temperature ammonia gas process to chemically modify their surface and thus increase their performance (e.g. faster start-up and higher current densities in MFCs, probably due to a more favorable adhesion of microorganisms to the positively charged anode). Other researchers developed improved anode materials, by impregnating them with chemical catalysts: for example Park and Zeikus (2003) used manganese modified kaolin electrodes, yielding power outputs up to 788 mW/m^2.

CATHODE: Similar to the cathode of a water electrolyzer, a MEC cathode normally consists of metal catalysts and catalyst-supporting materials. A catalyst is a substance that increases the rate of hydrogen production at the cathode surface by reducing the activation energy barrier considerably, thereby lowering the "cathodic activation overpotential" (see the next section within this paragraph). The reaction involved leaves the catalyst unchanged.

Platinum (Pt) is well known as the best catalyst material for this reaction and is commonly used in MEC systems: platinum catalyzed electrodes are commercially available or prepared in the laboratory by mixing platinum with a chemical binder (for example 5% Nafion solution or 2% PTFE solution; Logan *et al.*, 2008) and then applying this mixture to one side of the cathode base (for example carbon paper). The high cost of Pt and its being subject to be poisoned by chemicals such as sulfide, easily to be found in common wastewaters, are unfavorable factors to the scale-up of MECs and rouse the search of better and cheaper alternatives to platinum-catalyzed cathodes. Nevertheless, differently from water electrolyzers, MECs operate under complicated biological and chemical conditions (presence of bacteria, bacterial metabolites, pH buffers and medium nutrients) and therefore it's not easy to find cost-effective alternatives, able to guarantee performances comparable with Pt catalyst under realistic MEC operative conditions.

Recently, some good results were achieved, using non-precious (costs about one to two orders of magnitude lower than Pt) metal catalysts such as nickel oxide (Selembo *et al.*, 2009), nickel alloys (Hu *et al.*, 2009), tungsten carbide (Harnish *et al.*, 2009) and stainless steel. Compared to conventional platinum catalyst, stainless steel made the cathodic hydrogen recovery improve from 47% to 61% and the overall energy recovery from 35% to 46% (Selembo *et al.*, 2009). As for the metal-alloy catalysts, they normally exhibit better catalytic capability than that of their pure components, due to synergistic electronic effects among alloys, but they are also easily prone to reduction in mechanical stability (with related decreasing performance).

Another recent alternative is the development of low cost biocathodes (i.e., absence of an inorganic metal catalyst) that use bacteria as cathode catalysts for hydrogen evolution in MEC. To develop this biocathode, Rozendal *et al.* (2008) proposed to create an

electrochemically active culture by enriching a biofilm of hydrogen-oxidizing bacteria on the anode and then to reverse the polarity of the electrode to obtain an active biocathode.
In our study (Paper IV), graphite fiber brush non-ammonia-treated anode and three different cathodes (carbon base layer plus platinum or molibdenum disulphide or stainless steel) were used to compare performances of MEC fed with high-COD industrial wastewater. Despite better performances of Pt and substantial methane production in all the MEC used, MoS_2 proved to be a valid alternative to Pt at the cathode, much more affordable for pilot- or real-scale appliances.

- *Membranes*

MECs may contain or not a membrane, distinct architectures called two-chambers and single-chamber MEC, respectively. A membrane is used to create a chamber where the microorganisms degrading the organic matter are kept separated from the cathode (where the hydrogen is evolved), and at the same time to regulate the ionic flux between the chambers. This configuration minimizes hydrogen losses to microbes on the anode and in the liquid and prevents mixing of the hydrogen product gas with carbon dioxide from the anode (Logan *et al.*, 2008), thus maintaining the high purity of the H_2 gas evolved. Moreover, membrane functions as a separator to avoid any short circuit.
By now, four main membrane types are used: the cation exchange membrane (CEM), the anion exchange membrane (AEM), the bipolar membrane (BPM) and the charge mosaic membrane (CMM). With respect to the transport numbers for protons and/or hydroxyl ions and the ability to prevent pH increase in the cathode chamber, the ion-exchange membranes are rated in the order: BPM, AEM, CMM, CEM (Rozendal *et al.*, 2008). However, the more widely used membrane type is the CEM, or more specifically the PEM (proton exchange membranes, such as Nafion™), which would selectively

permeate the positive charges from the anode to the cathode chamber. During operation, however, cation species other than protons are responsible for the positive charge transport through the cation exchange membrane (Rozendal *et al.*, 2006b) because concentrations of Na^+, K^+, NH_4^+, and Ca^{2+} in wastewaters (≈ pH 7) are typically present at concentrations 10 times higher than the protons (Logan *et al.*, 2008). As a result, protons consumed at the cathode are not replenished by protons generated at the anode and this leads to a membrane pH gradient (the pH increases at the cathode and decreases in the anode chamber) which reduces MEC performance. Indeed, every pH unit difference between the two chambers will increase E_{eq} by 0.06 V, which corresponds to an additional energy requirement of about 0.13 kWh/m^3_{H2} per pH unit (Logan *et al.*, 2008). Fan *et al.* (2008) reported that a Nafion™ membrane in a two-chamber MFC developed resistance up to 86% of total internal resistance.

As a solution, the anion exchange membrane, which allows for the transport across the membrane of negatively charged chemical buffers, such as phosphate and bicarbonate alkalinity, is progressively more widely adopted. It has been reported that AEM is better than CEM, as it helps to buffer pH changes between the two chambers and thus to substantially increase MEC performance (Cheng and Logan, 2007; Rozendal *et al.*, 2007).

Alternatively membranes could also be avoided, because, contrarily to water electrolyzers where they are required to prevent the explosive mixtures of oxygen and hydrogen gases, there is no oxygen evolution/presence in a MEC. Removing the membrane reduces ohmic resistance and helps to reduce the bulk pH gradient in the liquid, although it does not prevent localized pH gradients at the electrodes (Logan *et al.*, 2008). Using a single chamber MEC with carbon cloth anode and carbon cloth loaded with platinum (0.5 mg

Pt/cm^2) for the cathode, Hu *et al.* (2008) produced 2.5 $mol_{H2}/mol_{acetate}$ at remarkable coulombic efficiency of 75% (with an E_{ap} of 0.6 V).

- *Overpotentials causing energy losses*

Compared to the maximal open circuit potentials (potential observed when no current is running through the electrical circuit), the real attainable voltage in a biologic cell is much lower, due to the so-called overpotentials, which are potential losses owing to electron transfer resistances and internal resistances (Figure 3.6). That's why, as previously stated, the voltage requirements to allow the hydrogen production at the cathode are considerably higher than the E_{eq}. Therefore, in order to reduce the hydrogen costs, it is important to minimize the irreversible energy losses as much as possible, while maintaining an acceptable hydrogen production rate (Logan, 2008).

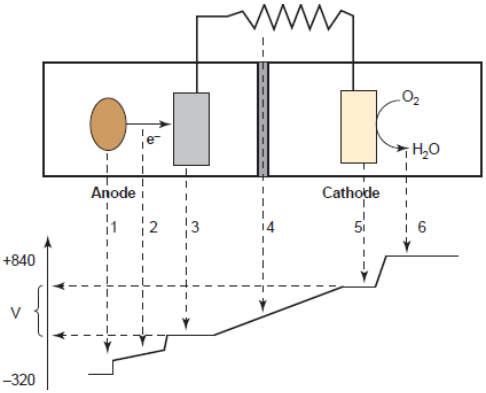

Fig 3.6 Potential losses during electron transfer in a MFC. 1. Loss owing to bacterial electron transfer; 2. Losses owing to electrolyte resistance; 3 Losses at the anode; 4. Losses at the MFC resistance (useful potential difference) and membrane resistance losses; 5. Losses at the cathode; 6: Losses owing to electron acceptor reduction (Courtesy of Rabaey and Verstraete, 2005).

Two main kinds of overpotentials can be defined, the electrodes overpotentials and the ohmic losses, and they both must be considered in order to calculate the actual energy requirements, E_{cell}, according to the equation:

$$E_{cell} = E_{emf} - (\sum \eta_a + \sum \eta_c + IR_{\Omega})$$

where $\sum \eta_a$ and $\sum \eta_c$ are the sum of the overpotentials at the anode and at the cathode respectively and IR_{Ω} is the sum of all ohmic losses within the system (Logan *et al.*, 2008). Each electrode can be treated individually (Wrana *et al.*, 2010) and among the parameters influencing the electrode overpotentials there are the electrode surface, the electrochemical characteristics of the electrode, the electrode potential, and the kinetics/mechanism of the electron transfer.

According to Logan *et al.* (2006), overpotentials in MECs are classified as:

1. *activation losses*: losses related to the transfer of electrons to or from a substance reacting at the electrode surface (such as the electron transfer mechanism between microbe and electrode at the anode). Wrana *et al.* (2010) suggest to focus on improving catalyst reaction kinetics by increasing operating temperatures and reaction surface areas.
2. *coulombic losses*: Coulombic Efficiency (CE) is defined as the amount of electrons recovered as current versus the amount of electrons available in the substrate. Therefore coulombic losses (e.g. the amount of electrons not recovered as current) are mainly due to microorganisms, that use a variable amount of energy for their growth and metabolism. A good balance between bacterial energy gain and electrode (terminal electron acceptor) may limit these losses.
3. *concentration losses*: poor mass transfer kinetics may limit the supply or the elimination of each substance provided to the cell and involved in the reactions. Substrate flux to the biofilm, diffusional gradients resulting from improper mixing, or unbalanced ratios of oxidized to reduced species at the electrode

surface are all contributing factors that will result in potential losses (Wrana *et al.*, 2010).

The ohmic voltage losses (IR_Ω) are independent of current and are due to both resistance to electron flow through electrical conductors (electrodes and external circuitry) and resistance to ion flow through ionic conductors (electrolyte and membrane) (Logan *et al.*, 2006). The proposed solutions for decreasing ohmic losses are the reduction of electrode spacing, the increase of electrolyte conductivity, the selection of electrode and membrane materials with low resistivity and also an adequate mixing of the medium within the cell.

- *Microorganisms*

Compared to MFCs, little is known about the composition of the microbial communities in MECs (Logan *et al.*, 2008). However, the same exoelectrogenic bacteria (*Shewanella* spp., *Geobacter* spp. and *Pseudomonas* spp.) were found in thick biofilms grown on the cathodes of both MFCs and MECs (Liu *et al.*, 2005) and it is assumed that the same exoelectrogens act in MFC and MEC.

The study of exoelectrogens has emerged as a new subfield of microbiology and many research efforts have brought to discover new strains and enrich their presence from various environments, such as domestic wastewaters, ocean sediments and anaerobic sewage sludges. Liu *et al.* (2010) have listed the isolated exoelectrogens in diverse genetic groups, including four of the five proteobacteria (a, b, g and d), firmicutes, acidobacteria and actinobacteria (here presented in Table 3.4 together with the current densities produced and the specific MFC configuration adopted).

Tab. 3.4 Taxa of bacteria, current density generated and reactor configuration used in MEC experiments (Courtesy of Liu *et al.*, 2010).

Taxon	Microorganisms	Current density (A/m^2)	Cell configuration (catholyte)/anode material
α-proteobacteria	*Rhodopseudomonas palustris* DX-1	9.9*	Single-chamber/graphite brush
	Ochrobactrum anthropi YZ-1	0.71	Two-chamber (ferricyanide)/carbon cloth
	Acidiphilium sp. 3.2sup5	3.0	Poised potential§ of 150 mV/graphite felt
β-proteobacteria	*Rhodoferax ferrireducens*	0.031	Poised potential¶ of 200 mV/graphite rod
γ-proteobacteria	*Shewanella putrefaciens* IR-1	0.016	Poised potential¶ of 200 mV/graphite felt
	Shewanella oneidensis DSP10	0.013	Two-chamber (oxygen)/graphite felt
	Shewanella oneidensis MR-1	0.018	Two-chamber (oxygen)/graphite felt
	Pseudomonas aeruginosa KRA3	0.017	Two-chamber (ferricyanide)/graphite rods
	Escherichia coli K12 HB101	1.0	Single-chamber/graphite-PTFE anode
	Klebsiella pneumoniae L17	1.2	Two-chamber (unknown)/carbon felt
	Enterobacter cloacae	0.13	Two-chamber (ferricyanide)/carbon cloth
	Aeromonas hydrophila PA3	0.3 mA‡	Single chamber/unknown
δ-proteobacteria	*Geobacter metallireducens*	0.65mA‡	Poised potential¶ of 200 mV/solid graphite
	Geobacter sulfurreducens	0.065	Two-chamber (oxygen)/graphite sticks
		0.163–1.1	Poised potential¶ of 200 mV
		8	Poised potential¶ of 200 mV/solid graphite
		5	Poised potential¶ of 200 mV/dimensionally stable Anode (titanium over iridium and tantalum oxide)
	Desulfuromonas acetoxidans	0.005	Two-chamber (unknown)/solid graphite
	Geopsychrobacter Electrodiphilus strain A2	0.066	Poised potential¶ of 0.52 V/graphite sticks
	Desulfobulbus propionicus	0.03	Poised potential# of 0.52 V/graphite sticks
Firmicutes	*Lactococcus lactis*	0.03	Poised potential¶ of 100 mV/graphite felt
	Thermincola sp. strain Jr	0.2	Poised potential¶ of 500 mV/graphite
	Clostridium butyricum EG3	0.22 mA‡	Poised potential¶ of 0.2 V/unknown
	Thermincola ferriacetica Z-0001	0.4	Single-chamber/solid graphite
	Brevibacillus spp. PTH1	0.009	Single-chamber/graphite rod
	Desulfitobacterium hafniense DCB2	1.1	Single-chamber/treated graphite block
Acidobacteria	*Geothrix fermentans*	0.097	Poised potential¶ of 200 mV/graphite
Actinobacteria	*Propionibacterium freudenreichii* ET-3	1.2 mA‡	Poised potential¶ of 400 mV/carbon felt

*Calculated based on projected cathode area (7 cm^4). The surface area of the brush anode was 2235 cm^2.
‡Anode area not specified.
§Versus standard calomel electrode as reference.
¶Versus Ag/AgCl electrode as reference.
#Versus standard H_2 electrode as reference.
PTFE: Polytetrafluoroethylene.

Among them, some, such as *Shewanella* and *Geothrix* species, are reported to produce their own electron shuttles and therefore have the advantage to be positioned at a distance from the electrode and yet still transfer electrons to the electrode surface. On the other hand, an isolated strain of *Pseudomonas aeruginosa* capable of utilizing glucose and producing an electron shuttle was inefficient in converting glucose to electricity, probably due to the incomplete oxidation of glucose to fatty acids (Rabaey *et al.*, 2005).

Therefore, if pure culture are useful to evaluate the mechanisms of electricity production and the strategies for optimizing this process, mixed cultures could have the advantage of increasing MEC

versatility with respect to substrate utilization, of enhancing the system robustness (due to biological diversity) and of being more adaptable to wastewater treatment. It is also interesting to note that while some isolates from mixed cultures demonstrate electrochemically active properties, most exhibit lower current densities when grown as pure cultures than as mixed cultures (Liu *et al.*, 2010).

Mixed cultures are usually enriched from domestic wastewaters and anaerobic sewage sludge (Liu *et al.*, 2005; Logan, 2008) or even from soils (Cheng and Logan, 2007) and have already been used in MEC studies (Call and Logan, 2008; Ditzig *et al.*, 2007; Rozendal *et al.*, 2006a; Tartakovsky *et al.*, 2009; Wagner *et al.*, 2009). The main practice for enriching a bacterial community for a MEC (start-up procedure) is to operate a MFC and then directly transfer the anode into a MEC: this procedure ensures the formation on the anode of a biofilm of suitable bacterial consortia and preselects an exoelectrogenic community for MEC operation (Logan *et al.*, 2008). During the start-up, the MFC, operated at low external resistance, will initially generate low current during biomass build-up, and hence will achieve a high anode potential and preferably select facultative anaerobes (Rabaey and Verstraete, 2005). Upon growth of the culture, the metabolic turnover rate, and hence the current, will increase. The moderate anode potential registered in this phase will favor, on the contrary, lower redox facultative anaerobes.

This procedure was also followed in our study (Paper IV).

Alternative practices for MEC acclimation is the use of effluents from working MFCs or MECs, full of active exoelectrogenic bacteria, or the creation of a solution made by suspension of biofilm scraped from the anode of an active cell (Cheng and Logan, 2007; Rozendal *et al.*, 2008).

Moreover, microflora enrichment directly in MEC is also possible (even if more risky), that has the advantage of direct bacterial adaptation to the operative reactor conditions.

Lastly, the complete absence of oxygen in MEC promotes not only the growth of obligate anaerobic exoelectrogenic bacteria, but also that of non-exoelectrogenic fermentative or methanogenic microorganisms, which could decrease the process performances (H_2 production and yield). Methanogens hydrogenotrophs are also favored by the presence of high concentrations of hydrogen gas within the MEC and could compete with exoelectrogenic bacteria for the substrate or use the hydrogen produced by them for converting it into methane. The suggested methods to inhibit the methanogens are:

- Avoiding buffer addition to the MEC (or at least no carbonate buffer). Rozendal *et al.* (2008) found that the use of a bicarbonate buffer with a biocathode encouraged the growth of hydrogenotrophic methanogens, able to use the buffer as a carbon source.
- MEC exposure to oxygen: Call and Logan (2008) showed in a membrane-less MEC, at an applied voltage of 0.6 V, that the exposure of the electrodes to air in between batch cycles reduced methane concentrations to < 1% in the product gas and did not impact current densities. In contrast, a lack of air exposure at the same applied voltage resulted in methane concentrations of 3.4% or more. The main problems of this strategy are that some substrates (e.g., cellulose) require strictly anaerobic conditions and that potential explosive mixtures (hydrogen + oxygen) could be created.
- Alternative strategies (to be investigated): preventing the methanogens growth by varying culture conditions, such as lowering pH, heat shocking of the inoculum, and short operation retention times (Logan *et al.*, 2008).

However, rather than to consider CH_4 as an undesired by-product, our study (Paper IV) tried to turn methane production in a MEC into a valuable result.

- *Feeding substrates*

Acetate is the typical carbon source for MEC, as it is the preferred substrate for exoelectrogenes, particularly *Geobacter sulfurreducens*. For example, MEC fed only with acetic acid achieved high H_2 recovery yields (91%) and production rates (1.1 $m^3_{H2}/m^3_{reactor\ (total\ volume)}$ d) (Cheng and Logan, 2007). However, in practice acetate generally does not exist alone and is typically found mixed with other organic acids (in wastewaters, landfill lecheates, and fermentation (acetate-rich) digestates). Thus, in the recent years, some studies have been made with simple substrates similar to acetate, such as butyric acid, lactic acid, propionic acid, and valeric acid (Cheng and Logan, 2007), while other studies aimed at treating acetate rich-wastes or diluted carbon sources found in wastewaters (Ditzig *et al.*, 2007; Wagner *et al.*, 2009).

Compared with fermentative hydrogen production, MECs can potentially utilize a wider variety of organic materials, because dark fermentation basically requires a fermentable (carbohydrate-rich) substrate, while MEC is subjected to theoretically less limitations. Indeed, bacterial respiration can occur on a wide array of substrates, ranging from pure compounds of sugars, carboxylic acids, alcohols and proteins to complex mixtures such as biomass hydrolysates, biowastes, agriculture residues and domestic, animal and food-processing wastewaters (Liu *et al.*, 2010).

However, using fermentable substrates such as glucose in MECs entailed several problems, as it resulted in increased diversion of electrons to nonelectricity sinks, such as biomass and organic byproducts, and in the reduction of exoelectrogens (in proportion to fermentative bacteria) in the community (Liu *et al.*, 2010). As a result, the kinetics of electron transport through the biofilm from

substrate to anode may be slowed, reducing the potential efficiency and the overall energy conversion efficiency of the system. Moreover, the use of a fermentable substrate may result in the accumulation of organic acids from fermentation, which lowers the pH of the reactor solution to as low as 4.5, a level that drastically reduces hydrogen production from a MEC, perhaps due to permanent damage to the exoelectrogens (Lu *et al.*, 2009).

Alternatively, cellulose was used for direct current generation in MECs using a mixed culture enriched from soil or a pure culture of *Enterobacter cloacae* (Cheng and Logan, 2007; Rezaey *et al.*, 2009). However, the current density generated from cellulose was ten-times lower than that from glucose and acetate. Therefore, pretreatment and hydrolysis, similarly to the pretreatment processes in conversion of cellulose to ethanol or fermentative hydrogen, may be needed to break down complex cellulosic biomass structures and to release soluble molecules, that can be more easily utilized by bacteria.

MEC can also use derivatives from sugar processing/fermentation include polyacolcohols, which can be found in the water-soluble materials of biomass, and uronic acids, which can be released from the hydrolysis of hemicelluloses (Liu *et al.*, 2011). Attention must be paid to some phenolic compounds, such as acetophenone and 3-4-dimethoxybenzyl alcohol, which have demonstrated strong inhibitory effects on exoelectrogens and must be removed.

Apart from issues about process performances optimization (H_2 yield and production rate), the choice of the substrate is fundamental also for making the process economically feasible. Indeed, taking into account sugar as feeding, 1 kg of sugar contains 4.41 kWh of energy and, considering that in the United Europe (EU) 1 kWh is worth up to € 0.16, even recovering all its energy content a maximum value of € 0.70 could be obtained, that is less than its market value (approximately € 1 in the EU). This means that, in order to produce enough hydrogen with MEC technology to supply the needs of

transportation and industry, it will be essential the use of an abundant, renewable biomass, preferably available on the market for low or negative (in case of waste) prices.

Therefore, new efforts have been recently dedicated to the study of wastewaters or waste biomass sources as sustainable substrates for the MEC process. In this way, a huge amount of the energy contained in the wastewater (it may amount up to 9.3 times the energy required to treat the waste with aerobic technologies according to Logan, 2004) may be recovered in a high value product (H_2), providing very high margins in profit and energy gain for a treatment process.

Easily digestible organic contaminants and some xenobiotics are metabolizable under microbial fuel cell conditions (Jang *et al.*, 2006) while particulate substrates have not been extensively studied in either MEC or MFC (Logan *et al.*, 2008). Focusing on actual wastewaters, different types have been tested as fuel in MFCs (Kim *et al.*, 2004; Liu *et al.*, 2004; Min and Logan, 2004; Min *et al.*, 2005; Oh and Logan, 2005; Yokoyama *et al.*, 2006; You *et al.*, 2006), while fewer studies with wastewaters have been conducted in MECs. MECs efficiently exploited domestic wastewater (Ditzig *et al.*, 2007) and swine wastewater (Wagner *et al.*, 2009) and hydrogen was also successfully generated, although at low rates compared to pure compounds in well buffered solutions, using winery and domestic wastewaters (0.17 and 0.28 m^3_{H2}/ $m^3_{reactor\ (total\ volume)}$ d, respectively; Cusick *et al.*, 2010). However, slow degradation of complex substrates and low conductivities of real wastewaters (typically 0.8 - 2 mS/cm) have been shown to decrease performance of MFCs and MECs, compared to tests developed under optimal laboratory conditions (Logan *et al.*, 2008). Thus, additional studies are needed about MEC using non-amended wastewaters (e.g. without nutrients or buffers addition). Therefore in Paper IV an industrial wastewater, with high-COD content and significant presence of methanol, was

used as feeding substrate without any amendment addition (buffer or NaCl). This work shows satisfactory COD removal and methanol exploitation in MEC, which has never been previously reported.

3.3 MEC application and scalability

Compared to other biofuel production processes, MEC shows interesting advantages from the applicative point of view:

- it directly generates high purity hydrogen, avoiding the production of undesired side products as in ethanol production from biomass;
- it doesn't require particular detoxification processes for the removal of inhibitory compounds (such as required in the ethanol-production process);
- it offers easy separation of high value product (H_2) from the liquid biomass solution, with reduction of separation/purification costs.

MECs are also advantageous for wastewater treatment because

- they generate less excess sludge in a more stable condition than the aerobic treatment process, where one-third to half of the operating costs are associated with solids handling and treatment (Kim *et al.*, 2007; Logan *et al.*, 2008);
- they can possibly limit the release of odors (Logan *et al.*, 2008).

All these aspects are promoting MEC application out of the lab-scale. However, since electrical energy is consumed in MECs, first of all they need to be more cost-effective than other existing technologies in the same field. Logan *et al.* (2008) compared aerobic (activated sludge, AS) and anaerobic processes (Anaerobic Digestion, AD) for wastewater treatment to microbial electrolysis (Table 3.5). These authors state that to be competitive, MEC must recover sufficient hydrogen from the wastewater and simultaneously minimize the required applied voltage.

Tab. 3.5 Energy requirements and production for wastewater treatment process.

Process	Volumetric Loading rate (kg_{COD}/m^3 d)	Sludge Production	Nutrient removal	Energy consumption (kWh/kg_{COD})	Energy production
Activated Sludge	0.5 - 2	high	Yes	0.7 - 2	No
Anaerobic Digestion	8 - 20	low	No	low	Yes, CH_4
Microbial Electrolysis	≈ 6.5	low (expected)	Possibly	0.5 - 2.4	Yes, H_2

MEC may have an energy consumption similar to that required for aerators in AS systems (0.5 - 2.4 kWh/kg_{COD}) and Rozendal *et al.* (2007) stated that full-scale MEC systems are expected to require ca. 1 kWh/m^3_{H2} and to produce 10 m^3_{H2}/m^3 d with a 100% overall hydrogen recovery efficiency. This means an energy consumption of 1.5 kWh/kg_{COD}, hence competitive with AS.

Moreover, compared to AS, they have the advantages of potentially treating higher volumes of wastewater (higher loading rate registered in continuous MEC system by Rozendal *et al.*, 2007) and of producing additional energy as hydrogen with lower excess sludge. In addition, MEC process (similarly to AD), developing in an environment not exposed to air as it is the AS, does not release odors: removal of chemicals associated with odors has already been demonstrated in MFC (Kim *et al.*, 2008). All these advantages potentially make MEC an environmental-friendly and healthy technology, acceptable by the media and the people.

Compared to AD, the first advantage of MEC is that from the same amount of COD, the gas produced (H_2) is more valuable than methane ($0.75/$kg_{H2}$ COD vs $0.11/$kg_{CH4}$ COD; Logan *et al.*, 2008). Capability of MEC for the nutrients (ammonia, nitrate, phosphorus,...) removal has not already been examined, but it could represent an additional advantage over AD process (Table 3.5). On the other hand, AD is already a well established and applied to real-scale technology which doesn't require any significant electrical energy input to produce methane. Moreover, CH_4 can be exploited by existing cheap technologies for electricity and heat production

(then easily sold or used for energetic self maintenance of the plant). However, it is also true that MEC is a new technology, whose performance can be greatly improved and design could be simplified. For sure, the commercialization of an efficient and scalable MEC system will depend on the full exploitation of the high-value gas produced (in order to compensate for the electrical energy costs of the system), on the adoption of an efficient design, applicable to real-scale, and on the cost effectiveness of a possible biomass pretreatment. Also, research is needed on whether MEC systems will be capable of stand-alone operation or if they will require aerobic effluent polishing step (as commonly is the case for ADs) (Logan *et al.*, 2008).

At present, while the high hydrogen yield by the MEC process has been demonstrated in laboratory tests, the scalability of MEC technology remains a challenge. No published detailed reports on MECs stand-alone pilot-scale plant are available. However, Logan (2010) reports the building of the first MEC pilot-scale plant (Figure 3.7), which has been conducted at the Napa Wine Company (Oakville, CA, USA) by Penn State researchers and engineers by Brown and Caldwell (Walnut Creek, CA, USA). The reactor design is based on the approach of immersing brush anodes and flat cathodes made of stainless steel into a tank (Call *et al.*, 2009; Logan, 2008; Selembo *et al.*, 2009). The reactor contains 24 modules, each with six pairs of electrodes, and is approximately 1 m^3 in total volume. No performance of this system are reported in the paper.

Fig. 3.7 Pilot-scale microbial electrolysis cell fed with winery wastewater at the Napa Wine Company in California, USA (Courtesy of Logan, 2010).

Another possibility for the scaling up of the MEC technology is connected to its ability to use the biodegradable components of a wide range of residual waste streams, even at very low COD concentrations such as reported by Kim *et al.* (2010a; 2010b). Therefore, the VFAs-rich effluents of dark fermentative or methanogenic (AD) bioprocesses could be used as feeding substrate for MEC, which may extract most of the chemical energy left in the effluents (otherwise representing a disposal burden and a waste of energy) and may also act as an effluent-polishing unit (Kim *et al.*, 2010a; 2010b). Therefore the association of a BES system to other biological technologies represents a good driving force for its real scale application. The combination of MEC technology with dark fermentative biohydrogen production stage will be dealt in details in section 4.3 of this PhD thesis.

Chapter 4

Biohydrogen and integrated energy production

By now, the main biological processing strategies able to treat complex substrates, such as industrial and agricultural wastewaters, and to simultaneously produce high-value products are the methanogenic anaerobic digestion, the biological hydrogen production, minor other fermentation processes and the bioelectrochemical systems. Biohydrogen production might be considered as more environmental-friendly process if associated to other technologies, which can exploit water, nutrients and by-products (mainly the VFAs) of the biohydrogen step for the production of fuels, energy or chemicals. In this way, the disadvantages of the dark fermentation stage (first of all, the low hydrogen yield theoretically achievable) may be overcome by the overall performances of a multi-stage process. This means that the H_2 stage can be potentially a major part of a complex (bio)factory

which includes pre-treatments, separations and multiple steps of biological or biochemical transformation of the organic matter. By producing multiple products, it is also possible to take advantage of the differences in biomass components and process intermediates and to maximize the value derived from the biomass feedstock.

Agriculture sector with its well established knowledge of bioprocesses, may play a leading role in this type of production complex, and therefore countries with large agricultural economies have potential for significant economic development through incorporation of bioenergy into bioindustry.

Some studies presented hydrogen as a potential by-product of other processes, such as gluconic acid production, an high-value biotechnology product (Woodward *et al.*, 1996). However this process, due to the low market of gluconic acid (about only 50,000 tons/year in the US), was not so economically convincing. This example helps to understand how one of the main problems of the multi stage strategies is the need to produce, together with biohydrogen, one or more high-value products for which there is also a large need, such as a fuel or electricity or a chemical.

In this Ph.D. thesis we present hydrogen production by dark fermentation of biomasses or wastewaters as the first stage of different possible "biofactory" stages, needed to counter the incomplete oxidation of organic matters achieved by the hydrogen stage and to recover the remaining energy. In this way the 85% of the energy still contained in the by-products of the dark fermentation stage (Logan, 2004) could be further and efficiently exploited.

The multi- (often two-) stage system also includes the idea that the overall biomass conversion is obtained through a sequence of biochemical reactions which do not necessarily share the same optimal environmental conditions (De Vrije and Claassen, 2005). Therefore every different fermentation steps, even conducted in physically separated reactors, must be optimized in order to generate

at the maximum reaction rate and yield the different valuable products of the process.

Finally, the choice of what reactions could efficiently been coupled must consider the overall process economy. For example, considering that the maximum hydrogen yield from glucose fermentation is 4 mol per mole of glucose (see section 1.3.2), paradoxically a doubling of the hydrogen yield can be achieved by fermenting 1 mol of glucose to 2 mol of ethanol and then reform the 2 mol of ethanol to 8 mol of H_2 (Deluga *et al.*, 2004). Thus, the synergy of the processes involved must be maximized in order to fully exploit the substrate, which otherwise fails to achieve complete conversion due to thermodynamic limitation.

In the next paragraphs, different conceptual integrated system configurations will be presented, even if this chapter doesn't pretend to include all the possible and near to infinite combinations of bioprocesses.

4.1 Dark fermentation + Photo fermentation

Sequential dark and photo fermentation is a rather new approach in biological hydrogen gas production. The conversion of the end-products of the dark fermentation stage is a process thermodynamically unfavored (ΔG_o = +75.2 kJ/mol), but phototrophic purple, non-sulphur bacteria are able to overcome this barrier by employing energy from light (*hv*) while using the organic acids (mainly acetate) as the prime carbon source, according to the following reaction (Claassen *et al.*, 2005):

$$CH_3COOH + 2\ H_2O + ‘‘hv’’ \rightarrow 2\ CO_2 + 4\ H_2$$

Specifically, in the first thermophilic dark fermentation stage, the biomass is fermented to acetate, carbon dioxide and hydrogen, while a separate second photobioreactor under anaerobic conditions converts the acetate formed in the first stage to additional hydrogen

(and CO_2), therefore reaching theoretical maximum production of 12 $mol_{H2}/mol_{glucose\ equivalent}$:

(i) Stage I - dark fermentation
$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2$

(ii) Stage II - Photofermentation
$2\ CH_3COOH + 4\ H_2O \rightarrow 8\ H_2 + 4\ CO_2$

Hydrogen gas production capabilities of (purple) photosynthetic bacteria such as *Rhodobacter spheroides, Rhodobacter capsulatus, Rhodovulum sulfidophilum* W-1S, *Rhodopseudomonas palustris, Rhodospirillum rubrum and Rodopseudomonos palsutris* P4 have been previously investigated, together with the range of organic acids (acetic, butyric, propionic, lactic and malic acid) which can be used by them as carbon source. Table 4.1 (Courtesy of Kapdan and Kargi, 2006) summarizes the yields and the rates of hydrogen production by photofermentation of different organic acids, as reported in literature. Figure 4.1 shows instead how this two-stage bioprocess could be physically realized, including also the possibility of gas recirculation in the first reactor (aiming at improving the H_2 recovery by gas sparging technique) and a gas separator system (for the upgrading of hydrogen, thus employable in end-use technologies like PEM-Fuel Cells). The major types of photo-bioreactors developed for hydrogen production are commonly tubular, flat panel and bubble column reactors, which have different features, especially if we consider the importance of photochemical efficiency parameter (theoretical maximum 10%). For example, reactors highly illuminated have lower light conversion yields but higher hydrogen production rates. On the other hand, they are more exposed to photo-inhibition of bacteria by excess light, potentially resulting in decreases in hydrogen production rate (Kapdan and Kargi, 2006).

Tab. 4.1 Yields and rates of biohydrogen production from organic acids by photo fermentation (Courtesy of Kapdan and Kargi, 2006).

Organic acid	Organism	Concentration	Light intensity	Conversion efficiency (%)	LCE[a] (%)	H_2 yield[b]	SHPR	VHPR	Process
Acetate	*Rhodopseudomonas*	22 mM	680 µmol photons/m^2 s	72.8	0.9			25.2 mL H_2/L h	Batch
	R. palustris	22 mM	480 µmol photons/m^2 s	14.8	0.1			2.2 mL H_2/L h	Batch
	R. palustris		2500 lux	60–70		2.8	9.8 mL/g cell h	1.6 mL H_2/L h	Batch
	R. capsulata	4 g/L	200 W/m^2	76.5		1.1	22 mL/g VSS h	0.88 mL/h	Batch
	R. capsulata	1.8 g/L	4170 lux	32.6	4.2		19.07 mL/g DW h		Batch
Lactate	*Rhodopseudomonas*	50 mM	680 µmol photons/m^2 s	9.6	0.4			10.7 mL H_2/L h	Batch
	R. palustris	50 mM	480 µmol photons/m^2 s	12.6	0.5			9.1 mL H_2/L h	Batch
	R. sphaeroides RV	100 mM	3klx	80			75 mL/g DW h	1.5 L/L d	CSTR
	R. capsulatus IR3	30 mmol	120 W	84.8					Batch
	R. sphaeroides GL-1	20 mM	300 W/m^2	86			0.2 mL/mL PU matrix h		[c]
Butyrate	*Rhodopseudomonas*	27 mM	680 µmol photons/m^2 s	8.4	0.3			7.6 mL H_2/L h	Batch
	R. capsulata	1 g/L	200 W/m^2	67.6		2.8	32 mL/g VSS h	1.28 mL/h	Batch
Malate	*Rhodopseudomonas*	15 mM	680 µmol photons/m^2 s	6.6				1.1 mL H_2/L h	Batch
	R. palustris	15 mM	480 µmol photons/m^2 s	36	0.3			5.8 mL H_2/L h	Batch
	R. sphaeroides	15 mM	200 W/m^2				2.4 mL/g DW h	12 mL/L h	Batch
	R. sphaeroides	7.5 mM	150–250 W/m^2	35–45			18 mL/g DW h	5 mL H_2/L h	Batch
PHB[d]	*R. sulfidophilum*		190 W/m^2					33 mL/L h	Batch
Succinate	*R. sulfidophilum*	50 mM	190 W/m^2					26.6 mL/L h	Batch

[a] Light conversion efficiency.
[b] H_2 yield mol/mol substrate.
[c] Immobilized on polyurethane foam.
[d] PHB, poly-hydroxy butyrate; 210 µmol photons/m^2 s = 190 W/m^2.

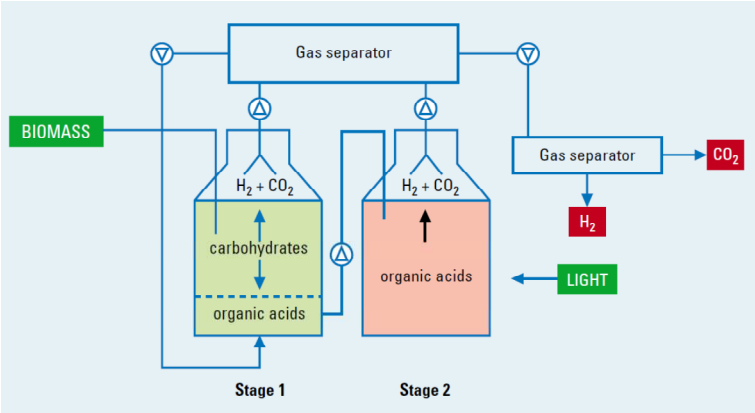

Fig. 4.1 Outline of a two stage fermentation process for hydrogen production from biomass. Stage 1: heterotrophic dark fermentation; stage 2: photoheterotrophic fermentation (Courtesy of De Vrije and Claassen, 2005).

Hereafter some of the most recent and relevant research experiences about a two-stage dark- and photo-heterotrophic bioprocess will be explored.

Kim *et al.* (2001) combined dark fermentation (using *Clostridium butyricum* NCIB 9576) with photo-fermentation (using immobilized *Rhodopseudomonas sphaeroides* E15-1 in hollow fibers) in a two stage process treating raw rice wine or Tofu (soybean curd) wastewaters (a carbohydrate-rich substrate). The dark stage had low HRT (1 day or less) and achieved 0.9 - 1 $L_{H2}/L_{wastewater}$ d, along with organic acids and ethanol production with rice wine waste. The effluent of this process was used for feeding the slower second stage (HRT of 10 days), where 0.44 and 0.2 $L_{H2}/L_{effluent}$ d were obtained with rice wine or Tofu wastewater, respectively. The authors reported also preliminary tests about mixing the effluent of the acidogenic stage with a pre-treated sewage sludge to increase the performance of the second stage.

Yokoi *et al.* (2002) reached high yield of 2.7 $mol_{H2}/mol_{glucose}$ in the first dark fermentative stage, by feeding the digester with mixed starch manifacturing wastes (sweet potato starch residue as a carbon source and corn steep liquor as a nitrogen source). Mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* HO-39 was used in this stage. The supernatant of the culture broth from the first stage was then used to feed *Rhodobacter sp.* M-19 culture, attaining hydrogen yield of 4.5 $mol_{H2}/mol_{glucose}$ (with pH adjustment at 7.5 and addition of EDTA and $Na_2MoO_4 \cdot 2H_2O$ to the medium). Therefore a very promising overall hydrogen yield (7.2 $mol_{H2}/mol_{glucose}$) was reached.

Nath *et al.* (2005) used glucose as substrate for a first dark fermentation step in batch (with pure culture of *Enterobacter cloacae* DM11), whose acid rich effluent underwent photo-fermentation by *Rhodobacter sphaeroides* strain O.U.001 in a column photo-

bioreactor, which constituted the second stage of the process. The dark fermentation stage reached yield of about 1.86 $mol_{H2}/mol_{glucose}$, while the yield in the photo-fermentation stage was about 1.5 - 1.72 $mol_{H2}/mol_{acetic\ acid}$, thus achieving an overall recovery of hydrogen of about 5 $mol_{H2}/mol_{glucose}$. The authors also observed that one of the main limitation of the process was the low light conversion efficiency of the second stage (which reached at maximum only 0.51%).

Also Tao *et al.* (2007) simulated a dark-fermentation stage through a batch test, where mixed microflora fermented sucrose and produced hydrogen and a mixture of fatty acids (mainly butyrate and acetate). The maximum hydrogen production rate was higher than 360 $mL_{H2}/L\ h$ and the maximum hydrogen yield was 3.67 $mol_{H2}/mol_{sucrose}$. Then photo-fermentation by *Rhodobacter sphaeroides* SH2C was employed to convert the fatty acids into more hydrogen, achieving a total hydrogen yield of 6.63 $mol_{H2}/mol_{sucrose}$ for the whole two-step process. Complete consumption of fatty acids was reported (no butyrate, acetate, propionate, or valerate were detected at the end of the process).

In 2008, Manish and Banerjee proposed a new process flow diagram (Figure 4.2) which would further complicate the system, introducing the idea of a three stage process where the effluent of dark-fermentation is sent to a photo-fermentation stage and that of photo-fermentation is sent to an anaerobic digester to produce biogas. Hydrogen and carbon dioxide gas mixture produced during the fermentation stages would be sent to a pressure swing adsorber for being upgraded.
The authors reported that this system reached higher H_2 yield than that of the single dark-fermentation process and reduced the requirement of sugarcane (in term of kg sugarcane input/kg_{H2}) approximately by 65% as compared to dark-fermentation. This lower

requirement also reduced the amount of electricity required during the milling process by 30%.

The process had the highest energy efficiency, the highest net energy ratio and lower greenhouse gases (GHG) emissions among different biohydrogen processes considered (photo or dark single stage fermentation systems or electrochemically assisted process). Compared to Steam Methane Reforming process, for each kg of hydrogen produced this system reduces GHG emissions by 7.31 - 9.37 kg CO_2 (≈ 57%) and non-renewable energy use by 123.2 - 148.7 MJ (≈ 65.79%) (Manish and Banerjee, 2008).

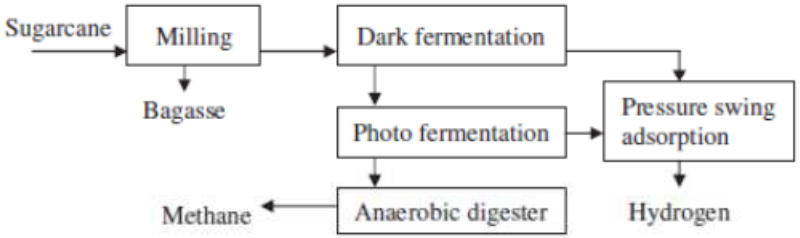

Fig. 4.2 Three stage fermentation process proposed by Manish and Banerjee (2008).

Aiming at real scale applications, a group of researchers (De Vrije and Claassen, 2005; Claassen *et al.*, 2005) designed a system consisting of a thermobioreactor (capacity of 95 m^3) for dark fermentation, followed by a photobioreactor (300 m^3) equipped with a sunlight collector (Figure 4.3). A preliminary system (extruder) was also used to exploit complex feedstocks as process substrates. Hydrogen was produced in the thermobioreactor by extreme thermophilic microflora (*Caldicellulosiruptor saccharolyticus*) and by *Rhodobacter capsulatus* pure culture used in the second stage. The size of the plant was set at a production capacity of 425 Nm^3_{H2}/h, which is equivalent to an energy production of 5.4 GJ/h, based on the upper combustion value of 12.74 MJ/Nm^3_{H2}.

Using glucose (derived from potato steam peels) as initial substrate, the process produced one third of the total hydrogen in the first stage (where it was recovered through gas stripping technique and further purified by pressure swing adsorption system) and the remaining two thirds in the following step. As a whole, the process achieved a conversion efficiency yield of 47%. This data is quite noticeable if compared with the maximum achievable efficiency of 69%, calculated assuming that the two separate fermentations may operate each one at 80% conversion efficiency.

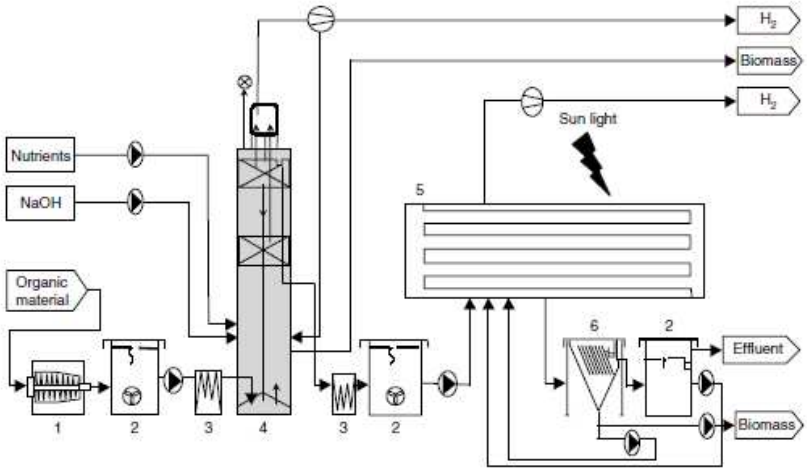

Fig. 4.3 Simplified flow sheet of the two stage hydrogen production bioprocess by Claassen *et al*. (2005). Details: 1: extruder; 2: tank; 3: heat exchanger; 4: thermoreactor; 5: photoreactor; 6: titled plate settler.

In conclusion, this two-stage production system has both advantages and disadvantages over single stage dark- or photo- fermentation processes.

Among the advantages, the limitation of the photo biological process requiring organic acids as substrates would be eliminated, considering the high acid content of the dark anaerobic process effluent. Further exploitation of its organic acids means also better

effluent quality in terms of COD. Most of all, higher hydrogen production yields can be obtained with the two systems combined and optimized.

On the other hand, there are many factors limiting the practical application of such a process.

A major challenge is the low photosynthetic efficiency, either with solar radiation or tungsten lamps, since at even moderate light intensities the main part (> 80%) of captured light is dissipated as heat (Hallenbeck, 2009). This involves the demand of large surface areas for anaerobic photobioreactors, which could be extremely expensive for practical applications. For example, Claassen *et al.* (2005) calculated that the required area of a photobioreactor converting the effluent of a 450 m^3 dark fermentation trickle bed reactor would be 12 ha. Therefore, photofermentative hydrogen production using current state of the art organisms and technology has been considered economically unrealistic by many authors (Westermann *et al.*, 2007).

Secondly, the system requires a continuous control in order to provide optimum media composition and environmental conditions for the two different microflora involved in the process. For example, dilution (also useful for reducing suspended solids, which decrease light penetration) and neutralization of dark fermentation effluents are required before feeding the photo-fermentation stage, in order to adjust the organic acid concentration and the pH to an optimal level for photosynthetic bacteria. Also the nitrogen content of the dark fermentation effluent must be monitored, since nitrogen, especially in the form of NH_4^+, not only inhibits the nitrogenase enzymatic activity, but also represses the synthesis of this enzyme fundamental for the photo-fermentation (Ntaikou *et al.*, 2010a). Establishing and maintaining nitrogen deficient environmental conditions or selecting/genetically modifying nitrogenase enzyme and its regulating system could be useful.

4.2 Dark fermentation + Anaerobic Digestion

The integration of a dark fermentative hydrolytic/acidogenic process with a subsequent, physically separated methanogenic process for combined hydrogen and methane generation, has been proposed by many researchers and shows many advantages, mainly an higher waste stabilization efficiency and an higher net energy recovery for the overall process (Angenent *et al.*, 2004; Ntaikou *et al.*, 2010a). Basically this strategy splits the traditional AD process in two stages, with the first step assigned to hydrogen and organic fatty acids production and the second to methanogenic (prevalently acetoclastic) fermentative reactions for methane production through acids consumption. These steps must occur in two physically separated reactors (as shown in Figure 4.4), providing the preferred environments for acidogenic hydrogenesis and methanogenesis (Han *et al.*, 2005). To this end, in order to reduce the characteristic differential between the two stages in hydraulic retention time (which in the second stage must be usually an order of magnitude higher than the first stage, due to the slower growth rate of the methanogenic archaea), many different reactors designs have been proposed, such as immobilized bioreactor (trickling filters and upflow anaerobic sludge blankets - UASBs -) for the AD stage (Guwy *et al.*, 2011).

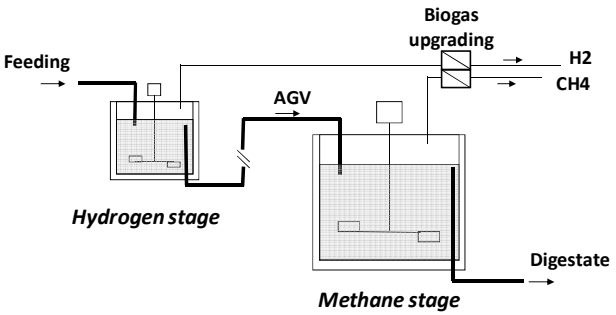

Fig. 4.4 Flow diagram of the two-stage (hydrogen production by dark-fermentation and methane production by anaerobic digestion) process.

The strictly methanogenic anaerobic digestion (AD) as a second stage is particularly advantageous because of its high organic removal rates, low energy-input requirement, energy production (i.e. methane) and low sludge production. Also, methane can be used as a fuel source for on-site heating or electricity production, or converted to other useful products, such as methanol for biodiesel production (Angenent *et al.*, 2004).

On my opinion, compared to the other strategies presented in this chapter, converting the organic matter remaining from the dark fermentative hydrogen step into methane gas also has the advantage to be the most feasible process for bioenergy production in the near term in real scale. Indeed, the technologies for methane production with simultaneous treatment of solid wastes and wastewaters already include single- and multistage processes and are well developed and established. This is particular true for our country (Italy), which in 2009 was Europe's number four biogas producer with 444.3 ktoe, and where it is expected the production of at least 2000 MWe from agro-energy plants within 2015 (Biogas barometer; Eurobserv'ER, 2010).

For real, this type of two-stage process has been traditionally suggested and used for merely improving the methane production and the AD process efficiency (Liu, 2008). Indeed, the separation of the process in two stages led in some studies to a larger overall reaction rate, organic matter conversion rate and biogas yield (Blonskaja *et al.*, 2003; Mata-Alvarez *et al.*, 1993). Also, the process achieved a better pathogenic destruction combining a short hydrolysis stage performed at thermophilic temperatures and a methanogenic stage at thermophilic or mesophilic temperatures (Bendixen, 1994).

However, the simultaneous production of two different gaseous energy sources/vectors (i.e. hydrogen and methane) brings new additional advantages to this process, from those intrinsically

connected to the H_2 (its energy content and environmental-friendly nature) to new ones deriving from the cooperation of the processes.
For example, stronger hydrolytic conditions typical of the first acidogenic step make the range of exploitable feedstocks wider: this strategy has been used so far for food wastes, cheese whey, olive mill wastewaters, household solid wastes, shredded paper wastes and wastewater sludges (Antonopoulou *et al.* 2008; Han and Shin, 2004b; Koutrouli *et al.*, 2009; Ntaikou *et al.*, 2010b).
Ueno *et al.* (2007b) reported that this kind of two-stage process achieves enhanced biological stability for wastes and higher organic loading rate capacities for the methanogenesis process, compared with the traditional single-stage process, while Hawkes *et al.* (2007) confirmed the general higher total efficiency on waste treatment and energy recovery than the traditional one stage process.
Moreover, recovering the hydrogen before bacteria turn it into methane, as it commonly happens in anaerobic digestion, makes the process advantageous also from economic point of view. Hydrogen gas ($6/kg) is more valuable than methane ($0.43/kg) and on the mass basis it also contains 2.2 times more energy than CH_4.
Logan (2004) assumed the possible monetary income deriving from the exploitation of a wastewater (2 g/L of BOD) from a single large food processing plant, generating 1.4 x 10^6 m^3/year of wastewater. He demonstrated that compared to the $310,000/year gained if all the BOD was just converted to methane, a two stage plant could produce $350,000 worth of hydrogen annually (assuming that all the organics in the wastewater were sugars and converted with a ratio of 2 $mol_{H2}/mol_{glucose}$), together with $260,000/year coming from the first stage effluent, fully converted into methane (assuming 0.4 L_{CH4}/g_{BOD}). Thus, at current gas prices the simultaneous recovery of hydrogen and methane would be economically more favorable than that of methane from a traditional AD process.

Another advantage is that the hydrogen-methane mixture is reported to significantly reduce air pollutants and to increase combustion efficiencies if adopted as fuel for internal combustion engines instead of pure methane (Bauer and Forest, 2001; Hallenbeck, 2009).
Figure 4.5 shows some of the inputs/outputs of the system, together with some mentions to operative aspects and advantages of the process.

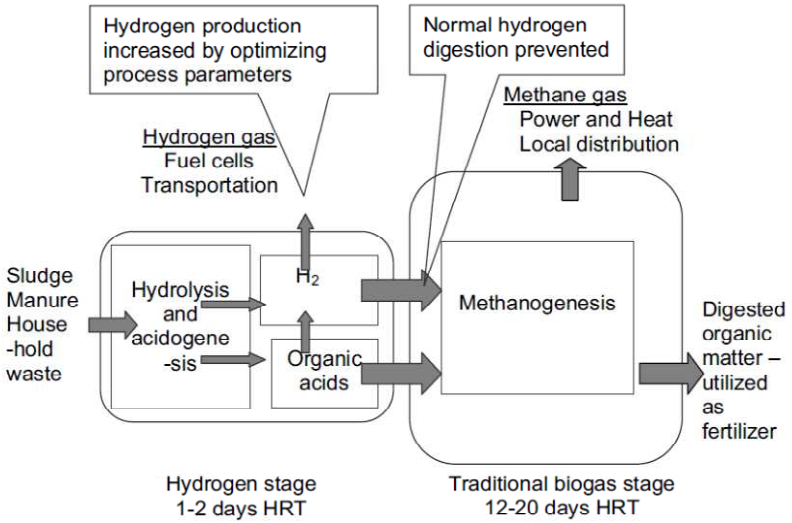

Fig. 4.5 Principle diagram of two-stage process for hydrogen and methane production (Courtesy of Liu, 2008).

Even the final outputs of the system (i.e. biogas and digestate) seem to possibly be positively affected by the separation in two stages. The biogas, a mixture of methane and carbon dioxide, is the gaseous end product of the organic matter degradation by anaerobic microorganisms and its quality depends on its composition, which usually consists of CH_4 (50-70%), CO_2 (30-50%) and smaller amounts of hydrogen sulphide and ammonia. Nevertheless, its composition strictly depends on the feedstock used as substrate, the

process conditions and the type of digester used. Beyond the advantages guaranteed by the two-stage anaerobic digestion, our study (Paper III) reports that the second methanogenic stage produce a biogas with a stably higher (> 70%) methane content than the typical range for AD process.

The digestate, i.e. the organic residue of the fermentation which shows a good content of essential nutrients (N,P and K), is the other main product of Anaerobic Digestion. Similarly to the one-stage process, also in the two stage system the digestate deriving from the methanogenic step can be split into a solid and a liquid fraction, with the first collecting the most of the phosphorous, and the second the most of ammonia-nitrogen. Thus, those fractions could be employed as fertilizer and as soil conditioner on farmland or gardens to improve soil quality. But in addition, in the two-stage process the digestate could be also used (instead of water) for achieving a better control on the reaction pH of the first stage and for simultaneously diluting the VFAs within the first bioreactor, thus warding off their inhibitory effect on hydrogen producing bacteria. Preliminary research was made by our group about this topic (Congress Communication II) but further research shall be carried out to test this strategy with different kinds of fermentable organic substrates and operational conditions.

Nevertheless, the two-stage hydrogen and methane strategy is not always advantageous: for example concentrated slurries and wastes with a high lipid concentration should preferably be treated in a one-stage digester (lipids will not be hydrolysed in the absence of methanogenic activity). Similarly, hydrolysis and acidification of proteins is not fully promoted by acidogenic conditions (de Vrije and Claassen, 2005).

Also, the two-stage strategy remains basically unproven in real scale as it adds complexity to the system and, as a consequence, increases investments and operational costs. Currently, just the 10% of full

scale biogas plants in Europe rely on two-stage (both methanogenic) processes (Choi *et al.*, 1997; De Baere, 2000).
Finally, the effect of increasing biogas production has not been accepted broadly, as separation of the two processes negatively affects syntrophic association and prevents interspecies hydrogen transfer between acidogenens/acetogens and methanogens (Reith *et al.*, 2005).

However, the scientific community made many researches in the last years about two-stage H_2-CH_4 anaerobic digestion process and some noticeable or new examples of them will be given in the next lines.

Liu *et al.* (2006) studied a two-stage hydrogen-methane fermentation process with household solid waste as substrate at mesophilic temperature, setting the HRT at 2 and 15 days for hydrogen stage and methane stage, respectively (Figure 4.6). The short HRT of the first stage was effective for separating acidogenesis from methanogenesis and no other control was used for preventing methanogenesis in the H_2 stage. This short HRT helped to maintain a stable pH (a key factor affecting the hydrogenic fermentation pathway) in the first bioreactor, where the optimum range for pH was found to be between 5 to 5.5.
The authors reported an hydrogen production of 43 $L_{H2}/kg_{VSadded}$ and a methane production of approximately 500 $L_{CH4}/kg_{VSadded}$. The overall methane production was 21% higher than one-stage process which was simultaneously run as control and which produced just 413 $L_{CH4}/kg_{VSadded}$. The same authors stated that these results were similar to other studies results, as those shown by Mata-Alverez *et al.* (1993) who achieved 510 L_{CH4}/kg_{VS} in a two-stage process for household solid waste fermentation and only 428 L_{CH4}/kg_{VS} in one-stage process (i.e. 19% methane increase). However, Mata-Alverez *et al.* (1993) didn't evaluate the first stage performance (e.g. H_2 production).

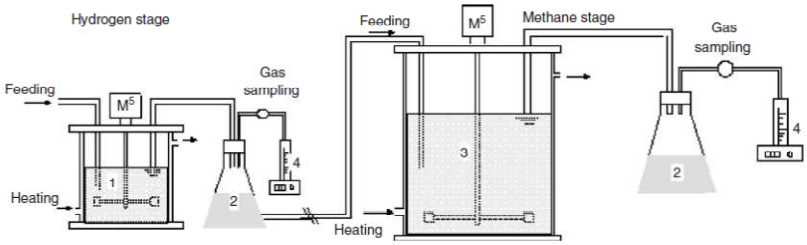

Fig 4.6 Schematic diagram of two-stage hydrogen-methane process by Liu *et al.* (2006). 1: Hydrogen reactor; 2: Effluent bottle; 3: Methane reactor; 4. Gas meter and counter; 5: Mixer.

Similarly, Paper III reports the comparison between a two-stage and one-stage AD process made by our group. Briefly, the hydrogen-stage (2 L working volume; 3 d HRT) of the two-stage thermophilic reactor gave relatively partial contribution to the total energy yield achieved by the system (13% of the total energy produced), according to previous works (Van Ginkel and Logan, 2005a). In our study the overall energy recovery doesn't show significant differences between the two- and single-stage AD systems (13-14 kJ/g $_{VS\ added}$), probably due to partial inefficiency of the methanogenic reactor (14.7 L working volume; 22 d HRT) of the two-stage process, but highlights the increase of the methane content for the methanogenic reactor of the two-stage system (≈ 70%), as compared to the single-stage process (55%).

Energy production shown in Paper III are also in agreement with results by DiStefano and Palomar (2010), who focused on the effect of two-stage process reactor configuration on the system energy (hydrogen and methane) yield. Five different reactor systems, all fed with a 40 ± 1 g/L complex synthetic substrate (Ensure media ® + micronutrients) and maintained at 35 °C, were investigated and compared: suspended growth, two-phase mixed, two-stage mixed, UASB reactor, and two-phase UASB. All reactor configurations achieved very high COD removals, on the order of 99%, but the highest energy productions, although lower than the maximum

theoretical value (15.2 kJ/g $_{COD\ removed}$), were reached by the mixed two-phase and two-stage configurations, amounting to 13.3 and 13.4 kJ/g $_{COD\ fed}$ respectively (Figure 4.7). The (two)phase or stage reactor configuration influenced microbial pathways in acidogenic reactors, since butyrate was the predominant volatile acid in phased configurations, whereas acetate was predominant in the staged configuration.

However, the idea suggested by these authors (DiStefano and Palomar, 2010), which is contrary to other studies, is that the complex substrates fermentation in a two-step AD process is justifiable primarily for improved process stability, improved methanogenesis performances and for the end-use zero GHG emissions technologies associated with H_2, whereas just secondarily for the total energy recovery point of view. Indeed, regardless of the reactor process configuration, hydrogen represented a minor proportion of the recovered energy input and theoretical analysis revealed that the maximum specific energy production from the two-phase suspended-growth configuration is only 9% higher than that from a single-stage mixed reactor (15.2 kJ/g $_{COD}$ versus 13.9 kJ/g $_{COD}$).

On the other hand, it is noticeable that the specific methane production for the two-phase and two-stage reactors was 22%-26% higher than that of the suspended growth reactor, which produced 0.30 L_{CH4}/g $_{COD\ fed}$. The authors justify this significant increment with the acidification achieved in the first stage, which has enhanced the biodegradability of the organic matter for the subsequent methanogenic reactor.

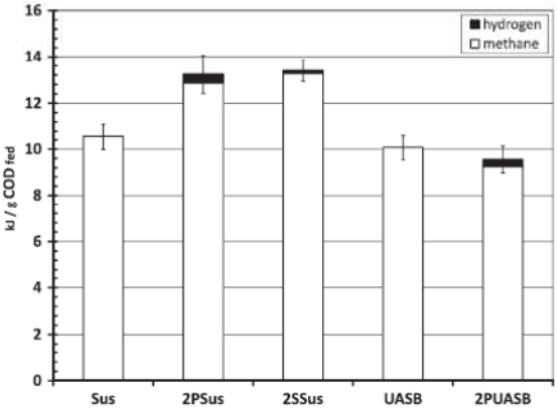

Fig. 4.7 Average specific energy production by process configurations (Courtesy of DiStefano and Palomar, 2010). Sus: suspended growth; 2PSus: two-phase mixed; 2SSus: two-stage mixed; UASB: upflow anaerobic sludge blanket; 2PUASB: two-phase UASB.

The laboratory experiment by Ueno *et al.* (2007b) aimed instead at evaluating the combination of a methanogenic process (with thermophilic down-flow packed-bed reactor) with a previous thermophilic (60 °C), pH-controlled acidogenic stage, alternatively used for hydrogenic or solubilizing operation. The first stage was fed with artificial garbage slurry containing milled paper. Hydrogenic operation resulted to be more suitable to combine with methanogenic process than solubilizing operation, since the retention time of the former was shorter (0.5 d) than that of the latter (4 d), nevertheless obtaining almost the same levels of overall removal efficiency in both COD and VSS. At 25 h of total retention time, with hydrogenic and methanogenic processes combined, overall COD removal and VSS decomposition were 82% and 96%, respectively. The process produced 199 $mmol_{H2}/L_{reactor}$ day (which means approximately a yield of 2.0 $mol_{H2}/mol_{\ hexose\ consumed}$) and 442 $mmol_{CH4}/L_{reactor}$ day (with an average content of 60% of CH_4 in the biogas). Comparing this two-stage system with single methanogenic process at the same HRT, again two times higher methane gas was produced and also

higher allowable OLR in methanogenic process was sustained (Ueno *et al.*, 2007b).

In the same year, Cooney *et al.* (2007) used a two-stage magnetic stirred hydrogen-methane system of 2 and 15 L working volume, respectively (Figure 4.8). This relative volume ratio (1:7) with a consequent short retention time in the methanogenic reactor was selected by the authors to test the assumption that separation of phase can enhance metabolism in the second reactor. Temperature and pH in both reactors were controlled and maintained at 35 ± 0.1 °C and at pH 5.5 in the first reactor and 7.0 in the second reactor through automated addition of chemicals. The reactor system was inoculated with conventional anaerobic digester sludge without pre-treatment (to simulate full scale operation) and fed with a glucose (10 g/L) - yeast extract (2 g/L) - peptone medium (2 g/L).
The authors showed that the selection pressure of pH and dilution rate is sufficient to select for acidogenic and methanogenic bacteria in their respective stages. The percentage of hydrogen in the head space of the first reactor was between 30% and 40%, regardless of varying dilution rates. However relatively low H_2 yields (between 0.05 and 0.35 $mol_{H2}/mol_{glucose}$) and rates (range: 11 - 85 $mmol_{H2}$/L d) were observed, probably due to an unfavorable predominant production of lactate (from 6 to up 31 g/L found in the first stage effluent). As for the second stage, after its preliminary independent operation, the integration into the two-phase system improved its CH_4 yields and production rates at all dilution rates, achieving up to 0.57 $mol_{CH4}/mol_{glucose}$ and 12.95 $mmol_{CH4}$/L d. However, the two stage integration did not show any accelerated metabolism in the second reactor that would allow the use of shorter retention times for it. Therefore, the functioning of a system with a small second stage reactor and with overall processing time shorter than usual (both of which would greatly decrease the cost of application in industry) was not supported.

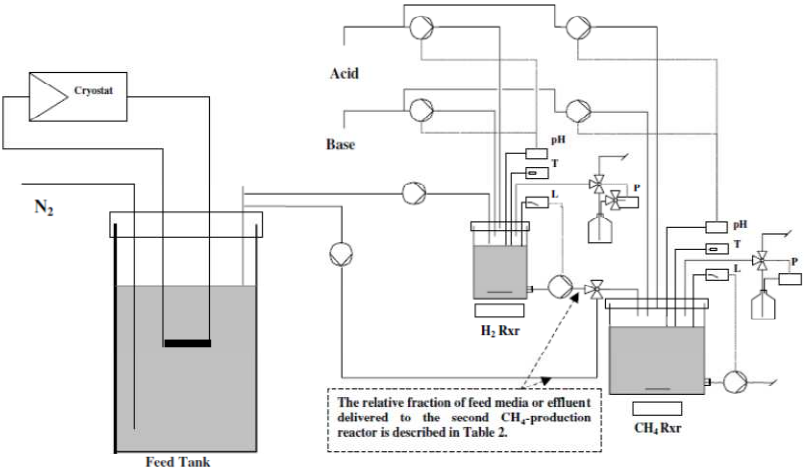

Fig. 4.8 The two-stage system design by Cooney *et al.* (2007).

Differently, Kraemer and Bagley (2005) focused on the effects of methanogenic effluent recycle in a two-phase anaerobic (Figure 4.9). The hydrogen reactor (2 L of working volume), fed with a synthetic glucose-rich medium at 37.3 kg_{COD}/m^3 d, was operated as a chemostat at 35 °C and pH 5.5 with a 10 h hydraulic retention time. The second stage was an up-flow reactor (12.5 L of working volume), operated at 28 °C and pH between 6.9 and 7.2. Without methanogenic effluent recycle, the H_2 productivity (0.115 $g_{H2\ COD}/g_{feed\ COD}$, data incorporating both gaseous and dissolved H_2) was higher than with recycle (0.015 $_{H2\ COD}/g_{feed\ COD}$), where presence of methane in the biogas up to 17% (v/v) was also detected. On the other hand, effluent recycle reduced the required alkalinity for pH control by approximately 40% and it could still be used without decreasing the yield, maybe through membrane filtration or heat treatment of the effluent, in order to exclude the return of hydrogen-consuming organisms. The improvement of this strategy could be particularly important for full-scale application of the two-stage AD process.

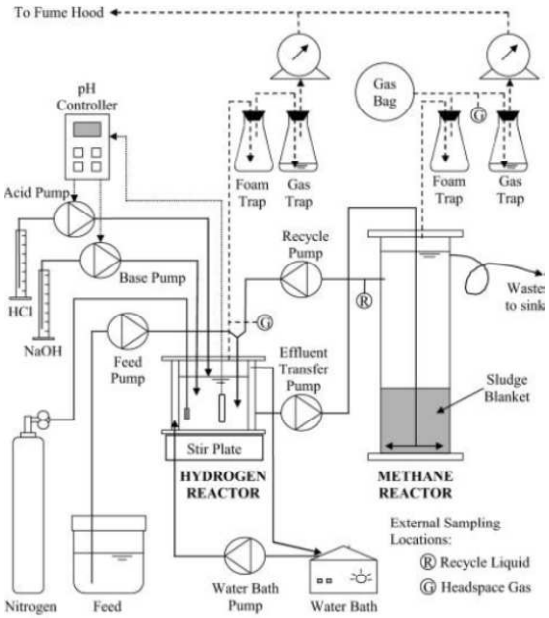

Fig. 4.9 Diagram of the experimental two-stage system of Kraemer and Bagley (2005).

Other minor examples of two-stage H_2-CH_4 producing reactors are reported in literature, and among them it could be interesting to mention of the BIOCELL system (Han and Shin, 2004b), due to its innovative design based on phase separation, reactor rotation mode, and sequential batch technique. The BIOCELL process consisted of four leaching-bed reactors for H_2 recovery, fed with food waste and operated in a rotation mode with a 2-day interval between degradation stages, and of one UASB reactor for CH_4 recovery trough the post-treatment of hydrogenic stage effluent. At the high volatile solids (VS) loading rate of 11.9 $kg/m^3_{reactor}$ day, this system could remove 72.5% of VS and convert the VS removed to H_2 (28.2%) and CH_4 (69.9%) in 8 days. H_2 gas production rate was 3.63 $m^3/m^3_{reactor}$ day, while CH_4 gas production rate was 1.75 $m^3/m^3_{reactor}$ day. The yield reached for H_2 and CH_4 were 0.31 and 0.21 $m^3/kg_{VS\ added}$, respectively.

However, although a good amount of laboratory two-stage fermentative systems have been reported, at the present there isn't any real scale application of this process and just few pilot scale experiences are known. A pilot plant for combined hydrogen and methane generation from a mixture of pulverized garbage and shredded paper wastes has been evaluated by Ueno *et al.* (2007a) (Figure 4.10). The first stage was a CSTR inoculated with thermophilic microflora enriched from excess activated sludge compost and kept at 60 °C, while the methanogenic stage was operated at 55 °C using an internal recirculation packed-bed reactor. The two stages had a HRT of 1.2 d (hydrogenesis) and 6.8 d (methanogenesis) and produced 5.4 $m^3/m^3_{reactor}$ d of hydrogen and 6.1 $m^3/m^3_{reactor}$ d of methane, with high chemical oxygen demand and volatile suspended solid removal efficiencies of 79.3% and 87.8%, respectively. Maximum hydrogen production yield was 2.4 mol/mol $_{hexose}$ and 56 L/kg $_{COD\ added}$. This study also showed that the methane yields were two fold higher than a comparable single-stage process.

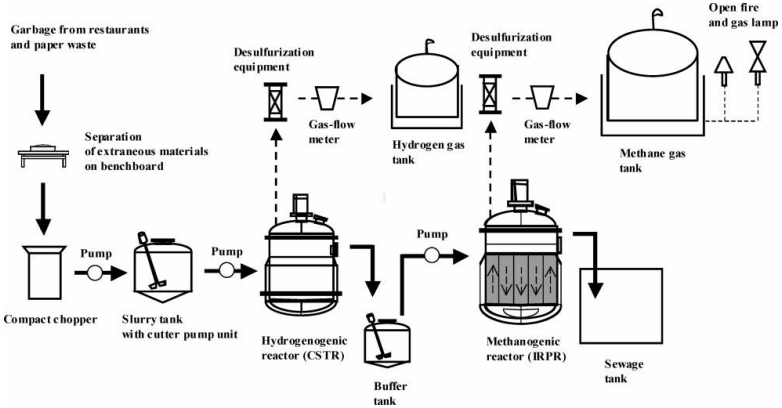

Fig. 4.10 Schematic diagram of the two stage pilot scale process by Ueno *et al.* (2007a). The arrows show the flow direction of raw material (solid lines) or biogas (dashed lines).

The Energy Technology Research Institute of the National Institute of Advanced Industrial Science and Technology in Japan operated a semi-pilot scale two-stage hydrogen-methane plant using wastes

collected from a local cafeteria: kitchen waste (50 kg/d), paper waste (3-5 kg/d) and food waste (10 kg/d). The plant configuration shows a solubilization/hydrogen fermentation tank of 1 m^3 capacity and a methane fermentation tank of 0.4 m^3 capacity (Figure 4.12). Few information are available about this plant, but the authors aim at reducing the overall process HRT from 25 to 15 days and at increasing both the decomposition of organic wastes from 60-65% to 80% and the energy recovery from 40-46% to 55% (AIST, 2004).

Fig. 4.11 Outer view of the semi-pilot scale two-stage plant by AIST.

Lastly, Wang and Zhao (2009) used a 0.2 m^3 rotating drum biohydrogen reactor integrated with a methanogenic CSTR of 0.8 m^3, fed with restaurant waste. They reported that 88% of the substrate was converted to biogas with a hydrogen yield of 0.065 m^3_{H2}/kg and methane yield of 0.055 m^3_{CH4}/kg.

Lee and Chung (2010) used a 0.5 m^3 mesophilic CSTR biohydrogen reactor fed with a supernatant produced from a ground, pressed and dehydrated food waste. The hydrogenic stage effluent was used to fed a 2.3 m^3 methanogenic UASB reactor operated at an HRT of 3.6 days and 36 °C for more than 40 days. The authors reported a hydrogen yield of 1.82 $mol_{H2}/mol_{glucose}$ and relate little difference in

the production costs between the single stage AD and the two-stage strategy, whose potential electricity generation was 10-12% more than that of single stage.

4.3 Dark fermentation + Bioelectrochemical systems

Due to the limitations of current technologies, acidogenic digesters for biohydrogen production and microbial fuel/electrolytic cells could hardly be used as sole system for wastewater treatment. Indeed, although the former is a good energy resource, its COD removal and energy efficiency remain low. Therefore, the new bioelectrochemical systems under development may be used for recovering the 85% of the energy that remains in wastewaters after hydrogen production by dark fermentation, in order to significantly enhance the overall hydrogen-production rate and yield (Hallenbeck and Ghosh, 2009), Otherwise, BES could act as a polishing step for the removal of the residual organics present in the effluent of the digester, which could still contain 0.5 to a few grams of residual volatile fatty acids (van Lier *et al.*, 2001).

The joint of these two bioprocesses into a two-stage treatment system may also represent a driving force for overcoming the practical limitations existing for BES application in real scale (due to high cost of the electrode materials, low or unstable materials performance and longevity at larger scale, low maximum achievable current densities, unfavorable wastewater composition...; Logan, 2010). For example, although BESs have low requirements for substrate specificity (Thygesen *et al.*, 2010), their process performances may be enhanced if the biomass used as feeding would be subjected to pretreatment or other treatment processes (which however must be cost effective in order to commercialize an efficient and scalable MEC system). Therefore, high-strength carbohydrate-

rich wastewater can be at first treated by dark fermentation to generate hydrogen and other metabolites favorable to be used in MECs, such as aliphatic acids and alcohols (Liu *et al.*, 2010).
The bioelectrochemical systems adopted after the first dark fermentative stage could be both microbial fuel cells (MFC) or microbial electrolysis cells (MEC) (Mohanakrishna *et al.*, 2010; Sharma and Li, 2010) and this choice could be based on that product (electric energy or hydrogen gas) thought to be more useful for a specific on-site application.

MFC or MEC connected with anaerobic fermentative hydrogen production processes has already been suggested (Pham *et al.*, 2006; Rozendal *et al.*, 2008), however, nowadays very few two-stage processes for the integration of these technologies are known or have been published.
Manish and Banerjee (2008) proposed a two/three stage process treating sugarcane biomass, where effluent of dark-fermentation stage is sent to a biocatalyzed electrolyzer to produce hydrogen and carbon dioxide (Figure 4.12). They assumed to use a two-chamber BES, in order to produce H_2 and CO_2 in different chambers and to avoid the use of pressure swing adsorption systems (or analogous) for the hydrogen upgrading. Compared to a single stage dark-fermentation process for biohydrogen production, the higher hydrogen yields in electrochemically assisted process led to lower need for sugarcane biomass input, thus increasing the energy efficiency of the process. On the other hand, this process had the least value of net energy ratio among the biohydrogen processes considered (photo or dark single stage fermentation and two-stage photo-dark fermentation), mainly because of significant electricity consumption by the electrolyzer. Improvements in the cell design and process parameters are required, in order to achieve less electricity consumption.

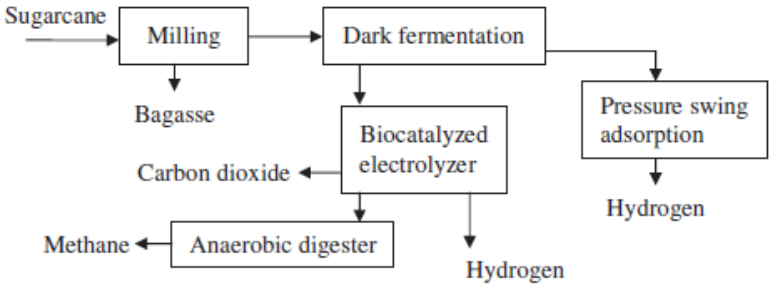

Fig. 4.12 Schematic diagram of a two/three stage process applying AD systems and a biocatalyzed electrolyzer (Courtesy of Manish and Banerjee, 2008).

The first studies that really exploited actual hydrogenic fermentation effluents for feeding MECs were those by Lalaurette *et al.* (2009) and Lu *et al.* (2009).

Lalaurette experimented a two-stage process for converting recalcitrant lignocellulosic material into hydrogen through a first thermophilic dark-fermentation stage followed by electrohydrogenesis, with optimization of the two steps in separate reactors. The lignocellulosic biomass was converted by the first stage into hydrogen, carbon dioxide, acetic, formic, succinic, and lactic acids, plus ethanol; then the residual volatile fatty acids (VFAs) and alcohols were transformed into hydrogen gas by Pt-catalyzed MEC. For the fermentation process, the authors used a pure culture of *Clostridium thermocellum* and achieved 1.67 $mol_{H2}/mol_{glucose}$ at a rate of 0.25 $L_{H2}/L_{reactor}$ d with a corn stover lignocellulose feed, or 1.64 $mol_{H2}/mol_{glucose}$ and 1.65 $L_{H2}/L_{reactor}$ d with a cellobiose feed. In the second stage, hydrogen yields and production rates from the fermentation effluents were 900 ± 140 L_{H2}/kg_{COD} and 0.96 ± 0.16 $L_{H2}/L_{reactor}$ d with cellobiose, while 750 ± 180 L_{H2}/kg_{COD} and 1.00 ± 0.19 $L_{H2}/L_{reactor}$ d with lignocellulose. Remarkable removal efficiency (70-85% based on VFA removal or 65% as for the COD removal) were obtained by MEC fed with the cellobiose and lignocellulose effluent. In particular, the overall hydrogen yield of the process was

9.95 $mol_{H2}/mol_{glucose}$ for the cellobiose, a noticeable result near to the maximal theoretical yield of 12 $mol_{H2}/mol_{glucose}$, thus favoring the techno-economic feasibility of the process.

Lu *et al.* (2009) used the effluent of an ethanol-type dark-fermentation CSTR reactor fed with a molasses wastewater as substrate for a single-chamber MEC. The first stage was fed at an organic loading rate of 22.8 kg_{COD}/m^3 d and produced hydrogen gas at a maximum rate of 0.7 $L_{H2}/L_{reactor}$ d and yield of 0.017 g_{H2}/g_{COD} (equal to 0.27 mol_{H2}/mol_{COD}). Its effluent had a pH in the range of 4.5-4.6, and a COD of 6500 ± 120 mg/L, mainly constituted by ethanol and acetate in solution. Due to this low pH, the effluent was added to the MEC both buffered (pH 6.7-7.0) or not. At an applied voltage of 0.6 V, the MEC fed with buffered effluent achieved an overall hydrogen recovery of 83 ± 4%, with a hydrogen production rate of 1.41 ± 0.08 $L_{H2}/L_{reactor}$ d. So, considering also the fermentation system, the overall hydrogen recovery was 96%, with a production rate of 2.11 $L_{H2}/L_{reactor}$ d, corresponding to an electrical energy efficiency of 287%. Moreover, this two-stage process showed an electrical energy demand of only 1.12 kWh/m^3_{H2}, which is much less than that needed for water electrolysis (5.6 kWh/m^3_{H2}). Lastly, the authors reported that the addition of a buffer to the first stage fermentation effluent was critical to MEC performance, as there was little hydrogen production using unbuffered effluent (i.e. 0.037 $L_{H2}/L_{reactor}$ d at E_{ap} = 0.6 V and pH 4.5).

Another couple of studies were later published about two-stage Dark fermentation-MFC process.
Sharma and Li (2010) used as first stage a 2 liters biofermenter (HPB) at continuous flow (Figure 4.13), fed at different organic loading rates (OLR) by changing the substrate COD and the hydraulic retention time. The reactor had a mixed microflora enriched by soil inoculum, it was fed with glucose and its temperature and pH were kept constant at 30 °C and 5.5 using a

build-in control system. The authors demonstrated that the hydrogen yield by this first stage increased with the decrease of OLR, and reached the maximum value of 2.72 $mol_{H2}/mol_{glucose}$ at the lowest OLR of 4 g/L d. The effluent from the hydrogen producing fermenter was then fed to a single chamber 100 mL glass bottle MFC, with graphite fiber brush as anode and a carbon cloth containing 0.35 mg/cm^2 Pt as the cathode. The MFC power density increased with the increase in influent COD concentration and the highest power density and coulombic efficiency of 4200 mW/m^3 and 5.3%, respectively, were reached at 6.3 g_{COD}/L. In MFC the energy conversion efficiency reached the peak value of 4.24% at the OLR of 2.35 g/L d and then decreased with higher OLR. The combination of the two system increased the overall energy recovery and the COD removal of the system, which reached a maximum of 29% and 71%, respectively.

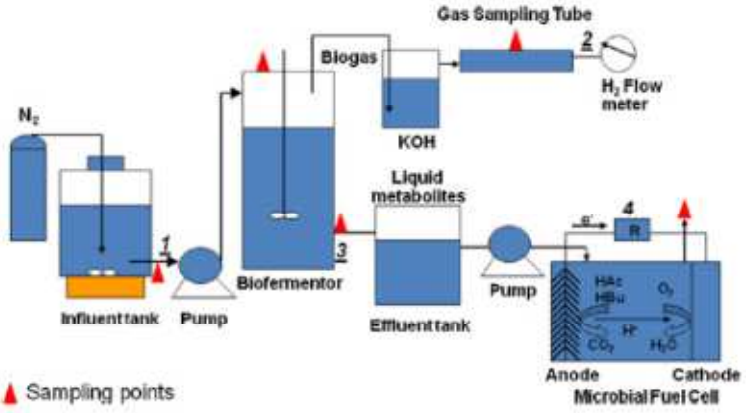

Fig. 4.13 Experimental setup of the continuous flow hydrogen fermenter plus the single chamber MFC by Sharma and Li (2010).

Mohanakrishna *et al.* (2010) integrated an acidogenic sequential batch biofilm reactor (AcSBBR), producing H_2 by fermenting vegetable waste, with a single chamber MFC which generated bioelectricity from the acid-rich effluents produced by the first stage

(Figure 4.14). AcSBBR was operated at high OLR (70.4 g_{COD}/L d) and a maximum yield of 2.93 mol_{H2}/kg $_{COD\ removed}$ was reached, showing an average COD removal efficiency of 28%. The effluent produced had an high average VFA content of 7713 $mg_{acetate\ equiv.}$/L (with acetate as the major metabolite (42%), followed by butyric acid (23.5%) and propionic acid (34%)). This effluent was chemically buffered to reach pH 7 and was then fed to the MFC at three variable organic loading rates. Higher power output (111.76 mW/m^2) was observed at lower substrate loading condition and MFC was able to efficiently remove effluent COD (80%), volatile fatty acids (79%), carbohydrates (78%) and turbidity (65%). Thus, the authors demonstrated the feasibility of utilizing acid-rich effluents by MFC for both additional energy generation and wastewater treatment.

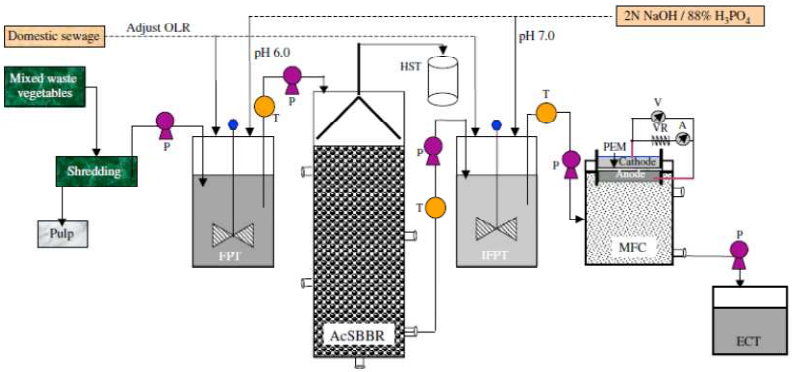

Fig. 4.14 Schematic experimental design of two-stage system by Mohanakrishna *et al.* (2010). AcSBBR: acidogenic sequencing batch biofilm reactor; FPT: feed preparation tank; IFPT: intermediate feed preparation tank/outlet of AcSBBR collection tank; ECT: effluent collection tank; MFC: microbial fuel cell; HST: H_2 storage tank; P: peristaltic pump; T: preprogrammed timer; V: volt meter; A: ammeter; PEM: proton exchange membrane; VR: variable resistor.

However, two-stage dark fermentation-BES systems technology need further research efforts and improvements to be applied in real scale.

4.4 Other approaches

- Dark fermentation + biopolymers production stage

In the chemical industry biomass can nowadays be fully exploited integrating in a single facility various processing options to produce simultaneously chemicals, fuels and energy.

Indeed, hydrogen production via dark fermentation could act as a pre-treatment step followed by a process of bioconversion of volatile fatty acids to other high added-value products, such as biopolymers like polyhydroxyalkanoates (PHAs). PHAs are biodegradable polyesters that specific bacteria can produce and accumulate under stress conditions as intracellular storage reserves (in the form of inclusion bodies/granules) of carbon and energy (Kessler *et al.*, 2001). There are several types of biopolyesters but poly(3-hydroxybutyrate) and poly(3-hydroxyvalerate) are the most well-known, since they bear similar structural properties to conventional plastics such as polypropylene, polyethylene and polyvinylochloride. They are also less penetrable to oxygen than conventional plastics, thus being ideal as packaging material in food industry or in many other applications of the medical sector. Therefore, PHAs may substitute the nowadays widely used petrochemical plastics, thus decreasing the environmental pollution due to anthropic activities (Ntaikou *et al.*, 2010a).

The production of PHAs from acidified wastewaters had been previously investigated in both lab (Dionisi *et al.*, 2005) and pilot (Kellerhals *et al.*, 2000) scale, showing very promising results. But it was just in 2010 that Ntaikou *et al.* (2010b) proposed a two-stage continuous system, which degraded a three phase olive mill wastewater and simultaneously produced hydrogen via dark fermentation in a CSTR and PHAs in an aerobic SBR using the first stage effluent. In the first stage they achieved hydrogen production rates between 165 and 202 mL/d (with a maximum yield of 196 $L_{H2}/kg_{\ consumed\ solids}$), while the second aerobic stage consumed

preferably butyrate to produce polyhydroxybutyrate, reaching a PHAs yield of 8.94% (w/w) of dry biomass weight. Following these promising results, the same authors lately reported the scale up of the process, conducting the two stage PHAs production at semi-pilot scale (Ntaikou *et al.*, 2010a).

The exploitation of low-cost substrates/wastewaters, such as the olive mill wastes used by Ntaikou *et al.* (2010b), together with the use of highly productive microorganisms (some of them can accumulate bioplastic up to 80% of their cell dry mass; Kim and Lenz, 2001) may be the right way to increase the overall economical viability of the process. However, issues concerning the separation of the soluble biopolymers from the fermentation broth and the stability of pure- or co-culture fermentation processes remain to be addressed. In particular, the downstream processing efficiency (separation and purification of the products from the bulk liquid) represents the highest percentage of the manufacturing cost, therefore one of the key goals is to adopt selective, efficient, and short separation routes.

- *The Maxifuel Danish Concept*

The Danish Bioenergy Concept is a combinatory system approach which can produce hydrogen, methane and ethanol altogether (Figure 4.15) (Westermann *et al.*, 2007). In this system biomass is pre-treated by wet oxidation to convert lignocellulosic compounds and to increase the availability of fermentable sugars, which are subsequently fermented to ethanol by yeast. Pentoses, which are not converted by yeasts, are then fermented again to ethanol and also to hydrogen in a (preferably) thermophilic fermentation process. Then the effluent of this stage is used to produce methane, and therefore the authors suggest to add it with manure as a cheap source of water and as a way to increase production of methane. The system also schedules to partly re-circulate the process water (after purification) into the methane step.

The advantage of this strategy is the creation of a close-cycle system and the possibility to specifically optimize each step for the production of the preferred energy carrier (hydrogen, methane, and ethanol) by choice of optimal microorganisms and operation conditions. Currently the Danish Bioenergy Concept is optimized with respect to ethanol production.

Fig. 4.15 The Danish Bioenergy Concept (Courtesy of Westermann *et al.*, 2007).

Chapter 5

Conclusions and final remarks

Hydrogen economy will be possible only with developments in existing technical and engineering challenges (H_2 upgrading, storage, transport and end-use technologies) and by lowering the costs and the sustainability of the present production strategies. Contrarily to the expensive, energy intensive and fossil fuel-demanding productive methods, our research efforts were dedicated exclusively to biological production of hydrogen via anaerobic (dark) fermentation of biomasses. Indeed, this biotechnology typically achieves high bio-hydrogen production rate and could be an economical strategy for waste disposal.

As first step, we managed to obtain efficient hydrogen-producing mixed microbial cultures directly from acclimation/enrichment of natural sources (soils and digested materials) (Paper II). Such type of inoculum, used in batch reactors with proper substrate and

metabolites concentrations, allowed H_2 yields comparable to those reached by more expensive pure/selected/GM microbial cultures.
Being the Dark Fermentation process influenced by the cost, availability and type of the raw material (biomasses) used, a new methodology (BHP test) was established and applied to test the biohydrogen production potential (BHP) of different organic substrates of possible interest for future real-scale applications.
Organic wastes/wastewaters from agriculture, livestock and food industry (in particular market bio-wastes, yielding 176 ± 2 NL_{H2}/kg_{VS}; Paper II) were proved to be attractive biomasses.
However, a concentrated mixture of organic fraction of municipal solid waste, although it showed very high and repeatable BHP in batch (202 ± 3 NL_{H2}/kg_{VS}), resulted in just 30% of its BHP when fermented in laboratory scale continuously stirred tank reactors (CSTRs), suggesting that further efforts are needed for future applications of dark fermentation in full-scale plants (Paper II).
Also co-digestion of wastes was proposed and applied to fermentative hydrogen production. Indeed, this process may suffer of inhibition or instability due to volatile fatty acids production and pH deviations from optimality. Therefore, the co-fermentation of promptly degradable feedstock with alkali-rich materials, such as livestock wastes, may represent a feasible and easy-to-implement approach to avoid external adjustments of pH. The natural buffer capacity of swine slurry was able to avoid pH drops in semi-continuous and continuous processes when fed together with fruit and vegetables residues. Optimal environment for high biohydrogen production rate and yield (3.27 $L_{H2}/L_{reactor}$ d, 126 $NL_{H2}/kg_{VS\ added}$) highly stable (deviations from daily average less than 14%) was maintained, without any chemical addition for process control (Paper I).

Simultaneously, multi stage system strategies were investigated, in order to extend past the present metabolic limitation of dark

fermentation (maximum yield 4 $mol_{H2}/mol_{glucose}$) and come closer to the 10 $mol_{H2}/mol_{glucose}$ required to approach costs competitive with traditional fuels.

Considering that the major part (85%) of the feeding substrate energy remains unused within the soluble compounds in the effluent of the H_2-producing process, association of this process with an anaerobic digestion stage for methane generation was tested. A two-stage laboratory-scale CSTR digester, fed with a mixture of agricultural and livestock residues, was monitored for a long run (approximately 700 hours) (Paper III). High hydrogen yields (140 $Ndm^3\ H_2\ kg^{-1}_{VS\text{-}added}$) were reached, with subsequent methane production of 351 $Ndm^3\ CH_4\ kg^{-1}_{VS\text{-}added}$. However, overall energy recovery similar to that of a single-stage reactor run as control was produced (13-14 kJ $kg^{-1}_{VS\text{-}added}$) and partial inhibition of the methanogenic reactor of the two-stage process was assumed. Nevertheless, biogas with high CH_4 content (≈70%) was produced, that is advantageous for lowering the biogas upgrading cost.

These results push to further in-depth researches about the two-stage process, that will be done both on laboratory scale continuous digesters and on a pilot scale two-stage reactor, located in the experimental farm of the University of Milan.

Lastly, initial investigations about bioelectrochemical systems for pure hydrogen biological production (MEC) were made. The variety of fuel sources and high yield of high-purity H_2 with just a relatively small electrical energy input make this process a promising approach and a new core technology for economically viable biohydrogen production, particularly from biomass with low or negative economic value. Furthermore if dark fermentation hydrogen production effluent may be used as feeding substrate for MEC.

Paper IV explored the rate and the yield of gas (for real a mixture of H_2, CH_4 and CO_2) produced by membraneless MEC exploiting an actual industrial wastewater with high methanol content, a compound

never before reported to be used in a MEC device. MEC energy recovery was positive (3.76 and 3.38 kWh/kg $_{TCOD\ removed}$ with platinum and molybdenum disulfide cathode, respectively) and 14-16% higher than an AD lab-simulated process. Also the TCOD removal efficiency was high (85-87%) with complete degradation of methanol. MEC emerged to be competitive with the AD process, especially using cheaper alternative catalysts such as molybdenum disulfide (MoS_2).

Starting from these preliminary remarks, a two- or three-stage reactor, which combines the technologies here introduced and studied (dark fermentation, bioelectrochemical system - MFC or MEC - and anaerobic digestion) will be realized and studied by our group within this year.

Chapter 6

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APPENDIX A

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Biohydrogen from thermophilic co-fermentation of swine manure with fruit and vegetable waste: Maximizing stable production without pH control

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ABSTRACT

Hydrogen production by dark fermentation may suffer of inhibition or instability due to pH deviations from optimality. The co-fermentation of promptly degradable feedstock with alkali-rich materials, such as livestock wastes, may represent a feasible and easy to implement approach to avoid external adjustments of pH.

Experiments were designed to investigate the effect of the mixing ratio of fruit–vegetable waste with swine manure with the aim of maximizing biohydrogen production while obtaining process stability through the endogenous alkalinity of manure.

Fruit–vegetable/swine manure ratio of 35/65 and HRT of 2 d resulted to give the highest production rate of 3.27 ± 0.51 L_{H2} L^{-1} d^{-1}, with a corresponding hydrogen yield of 126 ± 22 mL_{H2} $g^{-1}_{VS\text{-}added}$ and H_2 content in the biogas of $42 \pm 5\%$. At these operating conditions the process exhibited also one of the highest measured stability, with daily productions deviating for less than 14% from the average.

1. Introduction

In the last decade, scientific and societal interests in possible applications of hydrogen as an energy carrier have grown tremendously due to hydrogen's unique environmental features. Indeed, molecular hydrogen H_2 stores the highest amount of chemical energy per mass unit (HHV = 142 MJ/kg) and can release it by reacting with oxygen, i.e., air, virtually without any exhaust emissions in the atmosphere except for water. Interest in hydrogen energy applications are also associated with very high conversion efficiency (45–60%), obtained with fuel cells as well with new homogeneous charge compression ignition (HCCI) engines (efficiency close to 45%) (Edwards et al., 2007; Gomes Antunes et al., 2008).

Despite present technological limitations and challenges, hydrogen is considered to be a possible energy carrier of the future, and developing sustainable methods to obtain hydrogen from renewable sources, in substitution of current fossil-fuel based technologies, is paramount in order to fully achieve all the potential benefits and environmental sustainability.

A first significant contribution to this goal may come from biohydrogen, i.e., hydrogen produced via biological conversion of organic matter by microbial consortia. In particular, mixed culture fermentation of substrates such as organic waste or biomass appears to be one of the most promising approaches able to bring in the next future the distributed production of renewable hydrogen, sustainable also on smaller scales.

Biohydrogen production by acidogenic fermentation under mesophilic, thermophilic and hyperthermophilic conditions has been demonstrated for a number of organic substrates especially rich in carbohydrates, such as simple sugars (often used as a model substrate), starch, sugar beets and potatoes processing wastewaters, cheese whey, brewery waste, etc. Hydrogen yields obtained with these substrates typically range from 50 to 150 mL_{H2} g^{-1}_{VS}, depending on the biodegradability and complexity of the substrate, and operation mode (Li et al., 2008; Shin and Youn, 2005; Venetsaneas et al., 2009; Yokoi et al., 2002) while that range can easily exceed 200 mL_{H2} g^{-1}_{hexose} for solutions of pure simple sugars (Aceves-Lara et al., 2008; Fang and Liu, 2002; Lin and Cheng, 2006; Van Ginkel and Logan, 2005; Zheng and Yu, 2005).

The equilibrium of the fermentation process can be disturbed by many biochemical and operating parameters. When promptly biodegradable substrates are used as feedstock, one of the most frequent factors of process inhibition or instability is the rapid build-up of undissociated volatile fatty acids (VFA) accompanying hydrogen production by acidogenic consortia. This leads to the depletion of medial buffer capacity, directly affecting the pH, which is known to play a crucial role in maintaining metabolic pathways and bacterial communities in an optimal domain for biohydrogen production (Lay, 2000; Lee et al., 2002; Mu et al., 2006; Ye et al., 2007; Zheng and Yu, 2005). Indeed, even if significant production has been reported for a wider range of conditions,

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fermentation pHs from 5.0 to 6.0 are very often considered optimal to avoid both methanogenesis and solventogenesis and to enhance biohydrogen generation (Hawkes et al., 2002; Van Ginkel et al., 2001; Wu et al., 2010). pH levels lower than 4.5 are known to negatively affect the activity of hydrogenase enzyme (Dabrock et al., 1992; Van Ginkel et al., 2001), driving the microbial community toward other fermentation pathways; on the other hand, a neutral or weakly alkaline pH favors methanogenic and homoacetogenic microbes development and their direct consumption of produced hydrogen, which can therefore be avoided by guaranteeing weak acidity in the environment.

To maintain the conditions within an optimal range, pH adjustment with the addition of exogenous acids and bases is often proposed as a process control solution in laboratory conditions. On the other hand, this approach may not be optimal for large-scale transfers, and when looking for full-scale applications different strategies may be considered for maintaining acceptable chemical equilibrium in fermentation broth.

For example, livestock manure is an abundant source of alkali and nutrients for cell growth, thus representing an ideal co-substrate for fermentation with carbohydrate rich and promptly degradable materials. In intensive animal industry areas, livestock waste could then represent a primary and renewable co-substrate for biohydrogen production. In Italy, for example, the pig industry consists of 9.5 millions animals and is mainly concentrated in highly specialized districts. It can be estimated (data not shown) that in the Po Valley area, more than 10 million tons of swine manure are produced yearly on a territory of about 5000 km^2. Such waste density levels are a major cause of environmental and societal problems, when not properly managed.

Demonstrating the sustainability of using pig slurries as a renewable source for supplying the production of biohydrogen would disclose new opportunities in a high-grade energy vector chain, while treating large amounts of agricultural waste.

Only a few studies have addressed the potential use of swine manure as feedstock for biohydrogen production. When used as a single substrate alone, very little biohydrogen could be recovered from fermentation both at mesophilic temperatures (Wagner et al., 2009), as well as at hyperthermophilic temperatures, with production yields lower than 4 $mL_{H2}\ g_{VS}^{-1}$ (Kotsopoulos et al., 2009). On the other hand, hydrogen yield as high as 203 $mL_{H2}\ g_{hexose}^{-1}$ (Wu et al., 2009) and as 209 $mL_{H2}\ g_{hexose}^{-1}$ (Zhu et al., 2009) were obtained when swine manure was added to glucose, reinforcing the hypothesis that it is a suitable co-substrate to be fermented by a mixture culture in addition to a carbohydrate-rich, promptly hydrolysable material.

Fruits and vegetables waste could be massively available to appropriate waste management approaches, and they represent a form of highly degradable feedstock to be used for biohydrogen production. Indeed, batch experiments at mesophilic temperatures conducted on cabbage and carrots pulp resulted in a maximal yield of 62 and 71 $mL_{H2}\ g_{VS}^{-1}$, respectively (Okamoto et al., 2000), while a composite mixture of vegetables reached 89 $mL_{H2}\ g_{COD}^{-1}$ (Venkata Mohan et al., 2009b); lettuce and potato yielded 50 and 106 $mL_{H2}\ g_{VS}^{-1}$, respectively (Dong et al., 2009). High biohydrogen yields were also obtained with fruit waste such as sweet lime peelings extracts (Venkata Mohan et al., 2009a) and jackfruit peel (Vijayaraghavan et al., 2006), obtaining biohydrogen yields of 76 $mL_{H2}\ g_{COD}^{-1}$ and 198 $mL_{H2}\ g_{VS}^{-1}$, respectively.

The objective of this work was to study the use of swine manure as a co-substrate for biohydrogen production by the thermophilic fermentation of easily degradable and carbohydrate-rich materials, such as fruit and vegetable market waste. In particular, the study aimed to maximize biohydrogen production rates while obtaining process stability through the indigenous buffer capacity of manure, and avoiding the use of any external alkali source.

2. Methods

Experiments were conducted in semi-continuously operated reactors, without pH adjustment of the fermentative broth. A central composite experimental matrix was designed to evaluate the effect and interactions of the two co-substrates' mixing ratio and hydraulic retention time, and to determine the conditions allowing for the highest biohydrogen production rates and self-stability at thermophilic temperature.

2.1. Startup inoculum

The seed microflora used in this study was collected from a 10 L laboratory-scale reactor, producing hydrogen by digesting glucose and fruit wastes. The reactor had been continuously operating under thermophilic conditions (55 °C) for months, prior to the beginning of this study, showing a stable production of biohydrogen at an average rate of 0.9 $L_{H2}\ L_{ferm}^{-1}\ d^{-1}$, and with a H_2 concentration in the biogas of 45 ± 5% (v/v). No methane was detected during the operation. Before being used in the experiments, the startup inoculum was transferred into a stirred flask and kept in anaerobic, thermophilic conditions for 3–4 d without adding any feeding substrate. During this time, the gas production rate sharply decreased to insignificant levels (less than 0.05 $L_{biogas}\ L^{-1}\ d^{-1}$) and the prepared inoculum was considered ready to seed the experimental reactors. The total solids (TS) and volatile solid (VS) concentrations and the pH of the inoculum resulted in 25.1 ± 4.3 g kg^{-1}, 18.9 ± 3.6 g kg^{-1} and 5.45 ± 0.15, respectively.

2.2. Substrates

The feeding substrate was a mixture of swine manure (SM) and fruit and vegetable market waste (FVMW). SM was collected from four different pig farms near Milano (Italy) and filtered through a stainless steel sieve (US Mesh No. 10, sieve opening of 2.0 mm). FVMW consisting of raw fruits and vegetables residues (apples, pears, potatoes, zucchini, etc.) was collected at different dates from a municipal market in Milano (Italy), freshly shredded in a blender and immediately frozen at −20 °C to avoid feedstock acidification.

TS and VS of SM used for the experiments were 10.0 ± 1.0 g kg^{-1} and 8.1 ± 0.5 g kg^{-1}, respectively, while FVMW had a TS content of 133.0 ± 8.0 g kg^{-1} and a VS content of 99.8 ± 4.0 g kg^{-1}. SM and FVMW showed pH of 8.1 ± 0.20 and 4.60 ± 0.10, respectively, and total alkalinity (TA) of 10.7 ± 0.2 $g_{CaCO3}\ kg^{-1}$ and 3.5 ± 0.1 $g_{CaCO3}\ kg^{-1}$, respectively.

Before feeding the reactors, shredded FVMW was acclimated to room temperature and mixed with filtered SM, according to mixing ratios defined by the experimental design. No other ingredient was added to the FVMW + SM mixtures, which were then used as the feeding substrate.

2.3. Experimental design

A Box–Wilson central composite design (CCD) (NIST/SEMATECH, 2010) was applied to study the effect of two operating parameters (the controllable factors) on biohydrogen production and process stability (the experimental responses), and therefore to find the optimal region in which to operate the fermentation. The two operating parameters considered in the study are: (a) the ratio of FVMW/SM of the co-substrates composing the feeding material; and (b) the hydraulic retention time (HRT) of the fermentation.

In a CCD, the experimental values of each controllable factor are defined to be uniformly distributed around a centerpoint, according to factorial design levels coded from −1 to +1. These levels

are then augmented with star points that, in a two-factor CCD, are axially placed at a coded distance of $-\sqrt{2}$ and $+\sqrt{2}$ from the center of the design.

As a result, the mixing ratio of co-substrates and HRT were investigated at five levels, coded as ($-\sqrt{2}$, −1, 0, +1, $+\sqrt{2}$). The level code reflects the step change in the actual value chosen for the two operating parameters.

All the evaluated levels were arranged in nine different treatments, corresponding to nine combinations of mixing ratios with HRT values. Each treatment consisted of three replicated assays, except for the centerpoint treatment, which was replicated six times.

Two sets of experiments were designed applying the described methodology: a preliminary set A aimed toward an exploratory screening of the experimental domain, and a subsequent, more focused set B, aimed toward deeper insight into the process.

In the preliminary set A, selected ranges for factors were 10–70% for FVMW content in the mixture with SM, and 1.5–4.5 d for HRT, with a design centerpoint of [30%; 3 d]. The resulting investigated range for organic loading rate is from 6 $g_{VS}\ L^{-1}\ d^{-1}$ to 32 $g_{VS}\ L^{-1}\ d^{-1}$.

According to results of experiments conducted on preliminary set A, more focused ranges for factors were chosen when defining set B. In this second set, FVMW content in the mixture was investigated in the range of 15–55%, while HRT varied within 1–3 d, centerpoint of the design being [35%; 2 d]. The corresponding range for the organic loading rate is approximately from 13 $g_{VS}\ L^{-1}\ d^{-1}$ to 53 $g_{VS}\ L^{-1}\ d^{-1}$.

All the coded levels and corresponding real values of operating variables considered in the experimental design are summarized in Table 1.

2.4. Semi-continuous fermentation and operating procedure

The fermentation assays were carried out using 500 mL Wheaton batch serum bottles, with an operating volume of 300 mL. The reactors were run on a semi-continuous basis, with broth withdrawal and feeding addiction operated at least every 12 h.

The bottles were firstly filled with the prepared startup inoculum. Even if known to influence the duration of the lag phase in batch biohydrogen production, the initial pH of the inoculum was not adjusted with chemical ingredients, as its value (5.45 ± 0.15) was considered adequate to guarantee optimal conditions for biohydrogen fermentative production. Similarly, for the entire duration of the assays, the pH of the broth was not adjusted, allowing it to reach indigenous chemical equilibrium.

According the design, an appropriate amount of feeding substrate was added up to obtain the operating volume. The bottles were flushed with nitrogen gas for 60 s to establish anaerobic conditions. They were sealed using screw caps with rubber septa tightly fitted to sample gas bags (SKC, Flexfoil) to collect the biogas produced by each reactor. The bottles were placed in a Dubnoff shaker bath at 55 ± 1 °C operated at a reciprocating frequency of 100 cycles per minute.

The reactors were fed by supplying the substrate every 12 h, except for assays with HRT shorter of 2 d which were fed every 8 h. Feeding of reactors was accompanied by the concomitant withdrawal of an equal amount of digested effluent. The effluent was periodically sampled for chemical analysis aimed to characterize the fermentation broth and process operation.

After startup, each reactor was allowed to reach steady conditions, and since then, it was operated during a period of four HRTs (preliminary set A) or six HRTs (set B).

After each run, every reactor was cleaned and freshly re-inoculated for new assays.

2.5. Measurements and analytical methods

Feeding mixtures and reactors' effluents were characterized according to Standard Methods (APHA, 1998) in terms of TS and VS, chemical oxygen demand (COD), pH, total volatile fatty acids (TVFA) and total alkalinity (TA) content as shown in Table 2.

Biohydrogen production was calculated from volume measurements of gas accumulated in sample bags and by measuring its hydrogen content. Biogas composition was determined with a gas chromatograph (Agilent, Micro GC 3000A) equipped with two thermal conductivity detectors and two different columns. Hydrogen and methane were analyzed using a Molesieve/5A Plot column, with nitrogen as the carrier gas at a flow rate of 30 mL/min. The carbon dioxide content was analyzed using a different column (Alltech HP-Plot U), with helium as the carrier gas at a flow rate

Table 1
The experimental design used for the study.

Treatment	Coded levels for factors		Actual values of factors			TS (g kg^{-1})	SV (g kg^{-1})	OLR ($g_{VS}\ L^{-1}\ d^{-1}$)
	Substrates ratio	HRT	FVMW(% w/w)	SM (% w/w)	HRT (d)			
Preliminary set A								
1A	$-\sqrt{2}$	0	10	90	3	22.3[a]	17.3	5.8
2A	−1	−1	20	80	2	34.6	26.4	13.2
3A	−1	+1	20	80	4	34.6	26.4	6.6
4A	0	$-\sqrt{2}$	40	60	1.5	59.2	44.8	29.9
5A	−1	0	40	60	3	59.2	44.8	14.9
6A	−1	$+\sqrt{2}$	40	60	4.5	59.2	44.8	10.0
7A	+1	−1	60	40	2	83.8	63.1	31.6
8A	+1	+1	60	40	4	83.8	63.1	15.8
9A	$+\sqrt{2}$	0	70	30	3	96.1	72.3	24.1
Set B								
1B	$-\sqrt{2}$	0	15	85	2	30.0 (1.5)[b]	26.0 (1.0)	13.0 (0.5)
2B	−1	−1	22.5	77.5	1.25	41.0 (2.0)	36.0 (1.5)	28.8 (1.2)
3B	−1	+1	22.5	77.5	2.75	41.0 (2.0)	36.0 (1.5)	13.1 (0.5)
4B	0	$-\sqrt{2}$	35	65	1	58.0 (4.0)	52.0 (3.0)	52.0 (3.0)
5B	0	0	35	65	2	58.0 (4.0)	52.0 (3.0)	26.0 (1.5)
6B	0	$+\sqrt{2}$	35	65	3	58.0 (4.0)	52.0 (3.0)	17.3 (1.0)
7B	+1	−1	47.5	52.5	1.25	73.5 (5.5)	66.5 (5.0)	53.2 (4.0)
8B	+1	+1	47.5	52.5	2.75	73.5 (5.5)	66.5 (5.0)	24.2 (1.8)
9B	$+\sqrt{2}$	0	55	45	2	83.0 (7.0)	76 (5.0)	38.0 (2.5)

[a] Calculated from average composition of the two co-substrates.
[b] Data measured from three replicates. standard deviations in brackets.

Table 2
Main experimental results for biohydrogen production and process stability.

Treatment	Actual values of factors			Biohydrogen production				Methane production
	FVMW (% w/w)	SM (% w/w)	HRT (d)	Production rate ($L_{H2}\ L^{-1}\ d^{-1}$)	Yield ($mL_{H2}\ g^{-1}_{VS\_added}$)	H_2 content (%)	Production stability index [a]	Production rate ($L_{CH4}\ L^{-1}\ d^{-1}$)
Preliminary set A								
1A	10	90	3	0.13 (0.02)	22.8 (3.9)	0.5 (0.5)	0 [b]	0.17 (0.03)
2A	20	80	2	0.24 (0.08)	18.4 (5.7)	13.2 (2.7)	0	0.65 (0.16)
3A	20	80	4	0.26 (0.04)	39.8 (5.9)	20.9 (1.3)	0	0.05 (0.04)
4A	40	60	1.5	2.82 (0.67)	94.5 (22.4)	51.9 (2.9)	0.61 (0.33)	0
5A	40	60	3	2.23 (0.07)	149.5 (6.4)	33.5 (1.4)	0.71 (0.03)	0
6A	40	60	4.5	0.14 (0.09)	14.5 (8.2)	14.5 (2.3)	0.25 (0.25)	0
7A	60	40	2	1.36 (0.50)	43.0 (15.9)	31.5 (5.9)	0.91 (0.04)	0
8A	60	40	4	0.35 (0.35)	22.0 (22.0)	5.1 (0.0)	0.22 (0.02)	0
9A	70	30	3	0.00 (0.00)	0.0 (0.0)	0.0	0.26 (0.07)	0
Set B								
1B	15	85	2	0.08 (0.05)	6.4 (3.6)	2.1 (1.5)	0	0.55 (0.05)
2B	22.5	77.5	1.25	0.02 (0.01)	0.5 (0.5)	0.1 (0.1)	0	1.20 (0.49)
3B	22.5	77.5	2.75	0.28 (0.14)	21.6 (10.4)	13 (5.0)	0	0.25 (0.05)
4B	35	65	1	1.38 (0.22)	26.6 (4.3)	17.7 (3.2)	0.55 (0.24)	0
5B	35	65	2	3.27 (0.51)	125.8 (22.4)	42.1 (5.1)	0.86 (0.04)	0
6B	35	65	3	2.06 (0.59)	119.3 (34.1)	37.6 (4.8)	0.77 (0.05)	0
7B	47.5	52.5	1.25	1.74 (0.82)	32.7 (15.5)	23.0 (8.2)	0.27 (0.04)	0
8B	47.5	52.5	2.75	2.39 (0.69)	98.9 (28.4)	41.3 (4.2)	0.67 (0.11)	0
9B	55	45	2	1.23 (0.63)	32.4 (16.6)	25.8 (8.0)	0.48 (0.41)	0

[a] See Eq. (1) for definition.
[b] Assays producing methane were assigned with a stability = 0.

of 30 mL/min. The operational temperature of the injection port was 100 °C, while those of Molesieve/5A and Plot U columns were maintained at 100 and 55 °C, respectively.

Biohydrogen production rate (P) was measured daily for each reactor, and for clarity, the values were normalized to the fermentation broth volume and then expressed as $L_{H2}\ L^{-1}\ d^{-1}$.

Biohydrogen yield was calculated as the specific production per VS mass added in each treatment and then expressed as $mL_{H2}\ g^{-1}_{VS\_added}$. An index for describing the production stability of a reactor was defined by considering the ratio of the standard deviation and mean of daily biohydrogen production and expressed as:

$$\text{Stability} = 1 - \frac{\text{sd}(P)}{\overline{P}} \quad (1)$$

According to Eq. (1), a reactor with constant hydrogen production has stability = 1, while production deviations as large as the average value are represented with stability = 0. Reactors producing a methane content in biogas higher than hydrogen were classified with a stability = 0.

In this study the terminal values measured during the last two HRTs of the specific treatment considered were assumed to be representative of the operative conditions investigated, i.e., the presented results of average and deviation for biohydrogen production rate, yield and stability of each assay were calculated considering the data measured during the last two experimented HRTs.

3. Results and discussion

3.1. Biohydrogen production rate and yield: effects of FVMW/SM ratio and HRT

The overall results obtained with all the experimental conditions considered in the study are summarized in Table 2.

As a general result, independent from the adopted HRT, low or no biohydrogen production was obtained in all the assays fed with a substrate having a FVMW content of 20% or less (i.e., SM $\geqslant$ 80%). In these cases (Treatments 1A–3A and 1B–3B), the average production rate of hydrogen was indeed always lower than 0.3 $L_{H2}\ L^{-1}\ d^{-1}$, with specific yields ranging from 0.5 $mL_{H2}\ g^{-1}_{VS\_added}$ to 40 $mL_{H2}\ g^{-1}_{VS\_added}$. Moreover, all these assays produced an increasing amount of methane, with terminal production rates even exceeding 1 $L_{CH4}\ L^{-1}\ d^{-1}$, indicating that a poor content of carbohydrate-rich material mixed with swine manure associated with a corresponding neutral pH of the feeding co-substrate, established fermentative conditions that are gradually far away from those required for optimal hydrogen production, but they are suitable for methanogenic bacteria development.

On the other hand, treatment 9A with 70% FVMW mixed with SM quickly resulted in large production instabilities, accompanied by drops of pH as low as 4.2 ± 0.2 (data not shown). In all the replicates of this treatment, the fermentation process resulted in a complete blockage before entering the two terminal HRTs, indicating an organic overload in the feeding. Low production also resulted in operating conditions corresponding to the longest tested HRT, that is, 4.5 d (treatment 6A), with less than 0.2 $L_{H2}\ L^{-1}\ d^{-1}$ for all the replicates and an average yield of 15 $mL_{H2}\ g^{-1}_{VS\_added}$.

In Fig. 1 the contour line charts of the experimental results for biohydrogen production rates of preliminary set A and set B, respectively, are plotted. With reference to preliminary set A, significant biohydrogen production rates were obtained in the experimental domain included in the HRT range 1.5–3 d and FVMW content in the co-substrate mixture in the range of 25–60%. Treatment 4A corresponded to conditions allowing the highest production rate of 2.82 ± 0.67 $L_{H2}\ L^{-1}\ d^{-1}$, reaching a peak of 3.21 ± 0.27 $L_{H2}\ L^{-1}\ d^{-1}$ for the best-producing replicate. This treatment, with a substrate composed of 40% FVMW and 60% SM, was associated with a fast HRT of 1.5 d, and it also produced the highest hydrogen content in biogas (51.9%), with an average yield of 95 $mL_{H2}\ g^{-1}_{VS\_added}$. Again, the same co-substrate but fermented with a longer HRT of 3 d resulted in the highest yield of 149 $mL_{H2}\ g^{-1}_{VS\_added}$, indicating the optimal balance in the mixture between carbohydrates from FVMW and the alkali/nutrients supply from SM at this mixing ratio.

As the highest producing condition [FVMW = 40%, HRT = 1.5 d] identified within preliminary set A resulted located on the border of the experimental domain, a second set B was designed with

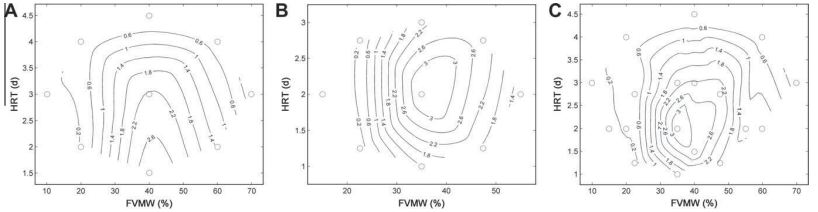

Fig. 1. Experimented conditions (○) and biohydrogen production rates contours obtained with data of preliminary set A (left), set B (middle) and overlapping set A + set B (right). Production data were normalized to the fermentation broth volume and expressed as $L_{H2}\ L^{-1}\ d^{-1}$.

the aim of investigating deeper insight into the neighborhood of that condition, as well as extending the study toward new conditions where possible high production could be found.

Overall biohydrogen production obtained with set B fairly overlapped with the results of set A (Fig. 1C). Nevertheless, the second design allowed for the identification of optimal conditions with a higher production rate than those found with set A. Treatment 5B (Fig. 2), with a FVMW content of 35% in co-substrate and a HRT of 2 d, resulted in $3.27 \pm 0.51\ L_{H2}\ L^{-1}\ d^{-1}$, with a peak of $3.76 \pm 0.51\ L_{H2}\ L^{-1}\ d^{-1}$ for the best-producing replicate and an average hydrogen content of 42 ± 5% in the biogas.

The overall highest yields in the study (120–150 $mL_{H2}\ g_{VS\_added}^{-1}$) were obtained with treatments 5A, 5B and 6B, i.e., in a close range of conditions corresponding to FVMW content of 35–40% and HRT of 2–3 d.

3.2. Response surface analysis of biohydrogen production

The results of biohydrogen production rate (P) were evaluated with a response surface analysis, a technique that determines whether the experimental response exhibits a significant curvature in its pattern and is therefore likely to have stationary points, where the optimal response is expected to occur.

To this aim, a second order model was introduced:

$$P = \beta_0 + \beta_1 \cdot \mathrm{FVMW} + \beta_2 \cdot \mathrm{HRT} + \beta_3 \cdot \mathrm{FVMW} \cdot \mathrm{HRT} + \beta_4 \cdot \mathrm{FVMW}^2 + \beta_5 \cdot \mathrm{HRT}^2 \quad (2)$$

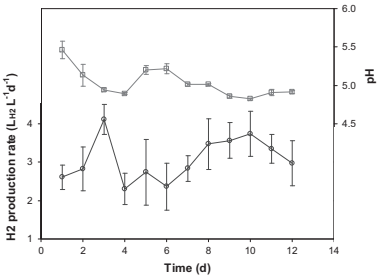

Fig. 2. Biohydrogen production rate (○) and pH of digested effluent (□) of the highest producing treatment (5B: FVMW = 35%, HRT = 2 d), mean and standard deviations for six replicates.

as a simplified surface function to locally approximate the production rate P obtained with a combination of the two independent variables (controllable factors), mixing ratio and HRT.

Coefficients β_i have to be determined by regression of experimental data, and once the adequacy of the fitting has been evaluated, the second order function (2) can be used to find the stationary points, representing a maximal (or minimal) response.

Statistical calculations have been conducted on centered variables fvwm* and hrt*, instead of using natural variables FVMW and HRT. Centered values were calculated by subtracting the mean value of the corresponding natural variable and normalizing to its step change (standard deviation). The results are expressed here in terms of natural variables, obtained after back conversion.

The analysis of variance for the fitting to Eq. (2) of experimental data of biohydrogen production rates, obtained with replicates of the two experimental sets, indicates that for both preliminary set A and set B, there is a significant curvature in the response surface. Indeed, all the quadratic coefficients (β_4 and β_5) for the two regressions have p-values < 0.001, while interaction between mixing ratio and retention time (β_3) does not have a significant effect on production with p-values much larger than 0.1.

In particular, for set B, the multiple correlations between all predicted and experimental production data resulted in a determination coefficient of $R^2 = 0.82$, with a RMSE = $0.50\ L_{H2}\ L^{-1}\ d^{-1}$. For this set, the best quadratic fit obtained after rejecting the non-significant interaction term (i.e., by setting $\beta_3 = 0$) was identified by the equation:

$$P = -14.13 + 0.52 \cdot \mathrm{FVMW} + 7.01 \cdot \mathrm{HRT} - 0.01 \cdot \mathrm{FVMW}^2 - 1.67 \cdot \mathrm{HRT}^2 \quad (3)$$

In Fig. 3, the experimental values of set B are plotted with the fitting function (3). By calculating the zeros of the derivatives, the location of the local maximum of the quadratic model can be found in correspondence to a FVMW content of 38.5% in the feeding mixture and a HRT = 2.1 d.

Regression of Eq. (2) was also studied when considering all the experimental data obtained with both preliminary set A and set B together. In this case, the multiples correlation between predicted and experimental production rates resulted in a lower determination coefficient of $R^2 = 0.69$, with a RMSE = $0.69\ L_{H2}\ L^{-1}\ d^{-1}$. The ANOVA for the fitting to Eq. (2) indicates that the linear dependence from HRT (β_2), as well as from interaction between mixing ratio and retention time (β_3) do not have a significant impact on production (p-values much larger than 0.05). Nevertheless, the quadratic model showed a lack of fit with the experimental data especially around the optimal range, with inacceptable underestimations of production rate larger than $1\ L_{H2}\ L^{-1}\ d^{-1}$, which excluded any use of the model to retrieve qualitative information about the process.

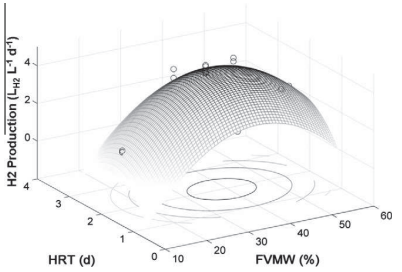

Fig. 3. Measured biohydrogen production rates (○) and the best fitting quadratic surface for experimental set B.

3.3. Biohydrogen production stability: effects of FVMW/SM ratio and HRT

The overall biohydrogen production stability summarized in Table 2 and illustrated in Fig. 4A and B. As they produced significant amounts of methane, treatments 1A–3A and 1B–3B (FVMW < 23%, regardless of HRT) were assigned with a stability = 0, even though some of them (2A and 3A) showed limited variability in their (low) hydrogen production, and (1A) showed a nearly constant lack of hydrogen production.

In the range of the experimental domain where significant biohydrogen production levels were obtained, the stability pattern overlapped with the production pattern. Indeed, the process exhibited a stability index greater than 0.7 for operating conditions corresponding to a substrate mixture containing 35–40% of FVMW and a HRT range of 1.5–3 d. Among highest production stability was obtained with the replicates of treatment 5B [FVMW = 35%, HRT = 2 d], with an index of 0.86 ± 0.04. A second peak was obtained with treatment 7A [FVMW = 60%, HRT = 2 d] which exceeded in stability the optimal range with an index of 0.91 ± 0.04. The replicates run under these conditions resulted then in the production of a stable and moderate (1.36 L_{H2} L^{-1} d^{-1} on average) biohydrogen volume.

3.4. Chemical parameters and process efficiency

Chemical characterization of set B took in consideration both the substrates and the digested effluents of the co-fermentation; Table 3 summarizes the obtained results. Substrate degradation accompanied the hydrogen production evidencing that the substrate had participated as a primary carbon source in the metabolic reactions involved in hydrogen generation. Effluent's COD ranged between 29 g_{COD} kg^{-1} and 85 g_{COD} kg^{-1} with a COD removal efficiency varying between 17% and 40%. The most productive experimental condition 5B showed an average efficiency in COD reduction of 32%, close to the highest value achieved by treatments 2B and 4B, both characterized by short HRT (1–1.25 d). COD consumption was relatively high also in treatments with a low H_2 production, but with a considerable CH_4 and/or CO_2 productions (1B, 2B, 4B).

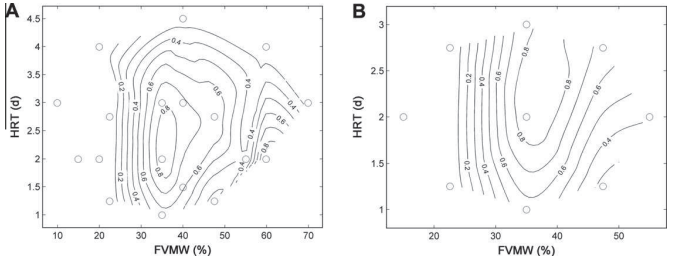

Fig. 4. Experimented conditions (○) and production stability contours obtained with data of preliminary set B (left), and overlapping set A + set B (right). Assays with a constant production of biohydrogen have stability = 1, while production deviations as large as the average value are represented with stability = 0. Reactors producing methane are classified with a stability = 0.

Table 3
Chemical characterization of substrates (IN) and digested effluents (OUT).

Trial	$pH_{\_IN}$ feeding substrate	$pH_{\_OUT}$ digested effluent	COD_{IN} (g_{COD} kg^{-1})	COD_{OUT} (g_{COD} kg^{-1})	COD removal (%)	$TVFA_{IN}$ ($g_{Acetic\ Acid}$ kg^{-1})	TA_{IN} (g_{CaCO3} kg^{-1})	$TVFA/TA_{IN}$	$TVFA_{OUT}$ ($g_{Acetic\ Acid}$ kg^{-1})	TA_{OUT} (g_{CaCO3} kg^{-1})	$TVFA/TA_{OUT}$
1B	7.71 (0.10)	5.64 (0.07)	41.3 (4.1)	28.9 (2.7)	30	1.76 (0.04)	9.21 (0.11)	0.19	11.2 (0.5)	8.3 (0.4)	1.35
2B	7.60 (0.15)	5.63 (0.32)	55.3 (3.7)	33.2 (6.2)	40	1.91 (0.07)	8.42 (0.98)	0.23	10.5 (410)	9.1 (0.3)	1.15
3B	7.38 (0.12)	5.40 (0.12)	55.3 (3.7)	45.9 (7.3)	17	1.91 (0.07)	8.42 (0.98)	0.23	11.4 (0.5)	8.4 (0.3)	1.36
4B	7.48 (0.14)	5.07 (0.23)	78.7 (5.2)	48.0 (4.4)	39	2.15 (0.07)	7.32 (0.12)	0.29	8.5 (1.1)	6.9 (0.7)	1.23
5B	7.35 (0.09)	4.88 (0.05)	78.7 (5.2)	53.5 (6.3)	32	2.15 (0.07)	7.32 (0.12)	0.29	12.2 (1.5)	5.9 (1.4)	2.07
6B	7.15 (0.13)	4.74 (0.05)	78.7 (5.2)	60.6 (5.8)	23	2.15 (0.07)	7.32 (0.12)	0.29	12.9 (0.8)	8.2 (1.3)	1.57
7B	7.21 (0.15)	4.43 (0.30)	95.9 (9.6)	70.0 (6.0)	27	2.41 (0.11)	6.85 (0.09)	0.35	8.3 (1.0)	7.1 (1.3)	1.17
8B	7.04 (0.09)	4.34 (0.12)	95.9 (9.6)	76.8 (4.9)	20	2.41 (0.11)	6.85 (0.09)	0.35	11.8 (2.4)	7.2 (0.3)	1.64
9B	7.00 (0.05)	4.57 (0.03)	110.2 (14.2)	84.9 (10.6)	23	2.73 (0.04)	5.66 (0.07)	0.48	11.2 (1.3)	6.5 (2.1)	1.72

Effluents's pH (pH_{OUT} in Table 3) were found in the range 4.2–5.7 and fairly correlated to SM content in the substrate ($R^2 = 0.87$, $p < 0.05$). In particular, treatments 1B–3B showed the highest pH values (5.4–5.7) commonly assumed as sufficiently low for inhibiting methanogenic bacteria (Liu et al., 2008; Hwang et al., 2009), but in this study that pH range resulted associated to a moderate methanogenesis and a very poor H_2 production. Moreover, all the hydrogen-producing conditions (4B–9B) showed pH levels below 5.1 and, in particular, the highest producing treatment (5B) had a pH_{OUT} of 4.88 ± 0.05 (Table 3). Even if such a pH value is relatively lower than the optimum typically indicated by literature (Van Ginkel et al., 2001; Liu et al., 2008; Hwang et al., 2009), the process resulted in relevant hydrogen production rates and yields, remarkably without using any external alkali for pH control.

Effluents' pH values were also fairly related to the endogenous alkalinity (TA_{IN}) and the TVFA/TA_{IN} ratio of substrates, indicating that organic acids production was successfully equilibrated by the alkaline species initially contained in SM. On the other hand, feeding substrate's TA_{IN} as low as 7 g_{CaCO3} kg^{-1} resulted in effluents' pH_{OUT} lower than 4.6, likely contributing to reduce the biohydrogen fermentation efficiency.

The TVFA concentration in digested effluents ranged between 8.3 $g_{Acetic\ Acid}$ kg^{-1} and 12.9 $g_{Acetic\ Acid}$ kg^{-1}, a range which overlaps the values found in similar conditions by Hwang et al. (2010), after 18 h of ripened fruit fermentation. Lower $TVFA_{OUT}$ concentrations were found when shorter HRTs were applied (2B, 4B and 7B), likely due to more intense metabolites wash-out. As expected, the most producing treatments (5B and 6B) showed the highest $TVFA_{OUT}$ concentrations, with a TVFA/TA_{OUT} ratio even exceeding 2. For these operating conditions the chemical endogenous equilibrium between the initial TA and the TVFA produced during the fermentation was able to maintain stable and remarkable hydrogen production.

4. Conclusions

The experimental design identified as optimal operating parameters a mixing ratio for substrate composition of 65% SM and 35% FVMW and a HRT of 2 d. At these conditions, the natural buffer capacity of SM was able to avoid pH drops and to maintain an optimal environment for high (3.27 L_{H2} L^{-1} d^{-1}, 126 mL_{H2} $g^{-1}_{VS\_added}$) and stable (deviations from daily average less than 14%) biohydrogen production, showing the feasibility of fermenting carbohydrate-rich substrates while avoiding the need for external alkali in possible implementations at pig farms.

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Looking for practical tools to achieve next-future applicability of dark fermentation to produce bio-hydrogen from organic materials in Continuously Stirred Tank Reactors

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ABSTRACT

This study aimed at finding applicable tools for favouring dark fermentation application in full-scale biogas plants in the next future. Firstly, the focus was obtaining mixed microbial cultures from natural sources (soil-inocula and anaerobically digested materials), able to efficiently produce bio-hydrogen by dark fermentation. Batch reactors with proper substrate (1 g $L^{-1}_{glucose}$) and metabolites concentrations, allowed high H_2 yields (2.8 ± 0.66 mol H_2 $mol^{-1}_{glucose}$), comparable to pure microbial cultures achievements. The application of this methodology to four organic substrates, of possible interest for full-scale plants, showed promising and repeatable bio-H_2 potential (BHP = 202 ± 3 NL_{H2} kg^{-1}_{VS}) from organic fraction of municipal source-separated waste (OFMSW). Nevertheless, the fermentation in a lab-scale CSTR (nowadays the most diffused typology of biogas-plant) of a concentrated organic mixture of OFMSW (126 g_{TS} L^{-1}) resulted in only 30% of its BHP, showing that further improvements are still needed for future full-scale applications of dark fermentation.

1. Introduction

Hydrogen is widely recognized as a clean and efficient energy resource with high potential for the future. Instead of common chemical or electrochemical hydrogen production processes and among the less energy intensive biological approaches (Oh et al., 2003) the dark fermentation has high H_2-production rate (Ueno et al., 2001) and relies on renewable resources. Many studies demonstrated the possibility of coupling hydrogen production with the utilization of a variety of organic substrates including waste materials, such as municipal solid waste, industrial wastewaters and agro-industrial waste and this may simultaneously provide economic and environmental benefits, meeting the growing demand for renewable energy (Guo et al., 2010; Lay et al., 1999; Oh et al., 2003). In particular, a number of authors reported that the two-stage anaerobic digestion (AD) process, as compared to traditional single-stage AD, would drive to differentiate the biofuel production (bio-hydrogen and bio-methane), improve the overall biogas production yields and allow higher CH_4 concentrations in the biogas produced in the second stage, decreasing the biogas purification costs. However, today most of full-scale biogas plants in Europe rely on single-stage process and the two-stage technology remains unproven in the field (Liu et al., 2006; Fantozzi and Buratti, 2009). This is mainly because dark fermentation process stability and the maximization of bio-hydrogen production yields in the first stage are still uncertain.

Rarely maximized and repeatable yields were reported even in pilot-scale, especially when complex and varied substrates (i.e. waste, residues, etc.) are fermented in relatively highly-concentrated organic mixtures (>100 g_{VS} $kg^{-1}_{wet\ weight}$) and in simple, low-cost and readily applicable reactor designs, such as the continuously stirred tank reactor (CSTR). Several studies on hydrogen production from wastewaters or solid waste have been made under different conditions in batch or continuous bioreactors, investigating both physical and biological parameters involved in the process, to optimize H_2-production yields and rates. Hydrogen production performances were reported to be variable because of fermentation conditions such as pH, hydraulic or solid retention time, hydrogen gas partial pressure, concentration of acids, microbial community of hydrogen-producing bacteria and presence of methane-producing microorganisms (Khanal et al., 2004; Lin et al., 2007; Nandi and Sengupta, 1998; Van Ginkel et al., 2001).

More recently, important efforts were focused, also, on reactor design and operational strategies for improving the H_2 yields of continuously-fed systems, looking for future applications of this bioprocess (Ding et al., 2010; Wang et al., 2010).

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However, the vast majority of literature contributions do not deal yet with technical solutions readily transferable to full-scale systems, but they focus on introducing improved strategies relying on selected-microbial cultures, on feeding approaches based on low-concentrated and/or completely soluble organic mixtures, or on innovative reactor designs.

In particular, regarding the microbial community, most studies were carried out on pure cultures of isolated strains of anaerobic bacteria, such as Clostridium sp. Because of the ubiquitous nature of hydrogen consumers and interspecific hydrogen transfer reactions (Lay et al., 1999), Clostridium sp. or other pure strains gave relatively higher hydrogen yield than natural mixed cultures, that makes the former particularly suitable for maximizing hydrogen production in laboratory test. Nevertheless, since dark fermentation has been shown to have great potential as applicable process to produce biohydrogen from a variety of organic materials (Levin et al., 2004), the use of natural mixed microbial communities instead of pure ones appear to be the most appropriate choice for future real implementation of this bioprocess. Indeed, mixed inocula do not require substrate-sterilization or other procedures that permit to maintain pure cultures active and, additionally, they are easily adaptable to different organic substrates (Ueno et al., 2001). Previous authors demonstrated that H_2-producing consortia can be obtained from various environmental sources, such as soil, compost, sewage sludge and various fermented organic materials (Kyazze et al., 2006; Li and Fang, 2007), but few investigated the effectiveness of such natural inocula on hydrogen productions (Akutsu et al., 2009; Morimoto et al., 2004; Van Ginkel et al., 2001) as compared to pure/selected cultures. Furthermore, the theoretical H_2 yield obtainable by standard substrates (typically glucose with a stoichiometric yield of 4 mol H_2 $mol^{-1}_{glucose}$) were rarely closely achieved and hardly repeatable (Fig. 1), even if pure/selected and genetically modified microbial strains were used. Additionally, most studies were focused on mesophilic microbial communities, while fewer on thermophilic ones, even if these latter were reported to show more promising H_2 production yields, near to maximal theoretical values (Schröder et al., 1994; Van Niel et al., 2002).

Besides the type of microbial community, also, the substrate/metabolites concentration is a factor particularly worthy of attention, affecting the efficiency of both substrate utilization and hydrogen production activity (Lin et al., 2007) by microbial communities suffering of both substrate and product inhibition. In mesophilic batch tests, hydrogen-producing bacteria growth, hydrogen yield and hydrogen production rate were all reported to decrease with increasing added ethanol, acetic acid, propionic acid and butyric acid concentration from 0 to 300 mmol L^{-1} (Wang et al., 2008). This may be a problem when relatively concentrated organic mixtures (more than 100 g_{VS} kg^{-1}) are used, such as typically happens in full-scale biogas plants. In addition regarding bioreactor typology and process design/operation, readily applicative perspectives were rarely addressed in literature. Most contributions have dealt with interesting innovative processes (such as up-flow anaerobic sludge bed, expanded granular sludge bed, internal circulation reactors, etc.), which are normally used with relatively low-concentrated (<10 g_{VS} $kg^{-1}_{wet\ weight}$) and completely soluble carbon sources (glucose, molasses, etc.). Contrarily, today the most diffused full-scale AD process design is the continuously-stirred tank reactor (CSTR) operated in "wet" or "semi-dry" conditions to produce bio-methane, i.e., one AD stage fed with solid–liquid complex mixtures of 50–200 g_{VS} $kg^{-1}_{wet\ weight}$ (Fantozzi and Buratti, 2009).

In this framework, this research addressed the viability of immediate applications of dark fermentation by using existing full-scale bio-technological solutions. Specifically, a first objective was to define a systematic and easily applicable lab-scale procedure for maximizing H_2-yields obtained with mixed microbial cultures, possibly approaching the results reported for pure cultures. Secondly, to apply this procedure on different organic substrates which may be of practical interest for H_2 production at full-scale and to assess their bio-H_2 potential production (BHP) with a lab-scale simple test. Finally, to verify to what extent the BHP can be achieved by the currently most diffused full-scale biogas plant design, namely the CSTR. This with a view of the possibility to shift to two-stage AD process for producing bio-hydrogen and bio-methane, by adding an appropriate module to the classical biogas plant.

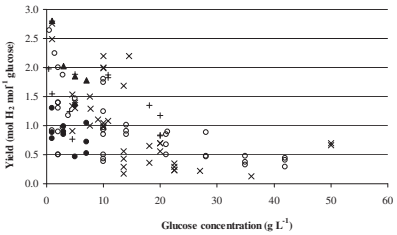

Fig. 1. Effect of substrate concentrations and type of inoculum on hydrogen yield: this work in comparison with literature results. (▲) acclimated inocula (this work), (●) soil inocula (this work), (○) literature results with naturally-sourced mixed microbial cultures, (×) literature results with pure/selected wild type microbial cultures and (+) literature results with genetically modified pure cultures (see Table S1).

2. Methods

2.1. Efficient hydrogen-producing inocula preparation from natural sources

The study was divided into two subsequent steps based on two different procedures of harvesting microbial consortia and preparing the fermentation environment.

In the first step, three different soils were used as sources of seed microorganisms: a rice soil (Inoculum A), a green urban soil (Inoculum B) and a vegetables-cultured soil (Inoculum C). These soils were dried for 24 h at 80 °C, shredded in a blender to pass through a mesh of about 2 mm and stored at 4 °C (APHA, 1998). The soils were suspended in water, getting a total solid (TS) content of the slurry of 70 g kg^{-1} wet weight (w.w.) (Table 1), then heat shocked at 100 °C for 2 h, in order to select spore-forming microorganisms and inhibit hydrogen consumers (Van Ginkel and Logan, 2005) and maintained for 4 weeks at 55 °C, under strict anaerobic conditions. The pH, total solids (TS) and the total volatile fatty acids (TVFA) were measured in all slurries, in order to characterize the fermentation environment. Batch anaerobic tests (55 °C incubation) were carried out with glucose as standard substrate (concentrations of 1, 3, 5 and 7 g kg^{-1}), both to compare the effectiveness of Inocula A, B, C and to find the ideal substrate concentration to maximize H_2 production yields (mol H_2 mol^{-1} glucose).

In the second step, a digested slurry (collected from a full-scale biogas plant treating household source-separated bio-waste mixed with agro-industrial by-products) was used as new source of microbial consortia. This anaerobically fermented material was treated, firstly, to inhibit hydrogen consumers (heat-shock at 100 °C for 2 h) and, secondly, to enrich the environment of fermenting microbial consortia. For this second purpose, the digestate was acclimated for a 4-weeks period in a laboratory-scale

Table 1
Naturally-sourced inocula characterization.

Inocula	Source	pH	TS (g kg^{-1})	TVFA ($g_{ACETIC\ ACID}$ L^{-1})
A	Rice field soil	6.7	70	<0.01
B	Soil from green urban area	7	70	<0.01
C	Soil from vegetable-cultured	7.2	70	<0.01
D	Harvested slurry (soil:acclimated slurry 1:1 w/w)	5.5	55	1.05
E	Harvested slurry (soil:acclimated slurry 2:1 w/w)	5.8	61	0.63
F	Harvested slurry (soil:acclimated slurry 3:1 w/w)	6	62.5	0.53

anaerobic bioreactor, fed with glucose solution (30 g L^{-1}) in semi-continuous mode (twice a day), with a hydraulic retention time (HRT) of 3 days and an organic loading rate (OLR) of 10 g glucose L^{-1} d^{-1}. This bio-reactor had a working volume of 0.6 L and was operated at a temperature of 55 ± 1 °C at a constant pH of 5.5 ± 0.5. The process showed a H_2 production of 0.96 LH_2 L^{-1} d^{-1} and H_2 concentration in the biogas of 45 ± 5% (v/v). No methane was detected during the test.

The digester output was then mixed with a blend of Inocula A, B and C (1:1:1 on w.w.), in order to decrease the high concentrations of metabolites observed in the digestate (TVFAs concentration of 2.1 ± 0.5 $g_{ACETIC\ ACID}$ L^{-1} during the 4-weeks observation) and at the same time to enrich soil inocula. Three different mixing ratios (1:1, 2:1, and 3:1 w/w between soils mixture and acclimated slurry) gave three new inocula (Inocula D, E and F, respectively) which were compared in batch bio-reactors, by feeding them with an equal substrate concentration (3 g glucose L^{-1}). The best performing inoculum was then tested with 1, 5 and 7 g glucose L^{-1}, in order to find the best methodology to test the bio-hydrogen potential of an unknown organic substrate through a batch test.

2.2. Bio-H_2 potential production (BHP) of selected organic substrates

Four selected substrates were tested by batch experiments to assess their bio-H_2 potential production (BHP): market bio-wastes (MBW), the organic fraction of municipal source-separated wastes (OFMSW), maize silage (MS) and swine slurry (SS). The batch tests were performed using the best inoculum harvested from natural sources and the same procedure developed in the previous tests.

The organic materials were collected from a full scale biogas plant near Milan (Italy), dried, shredded in a blender to pass into a 1 mm mesh and added to the bottles at the concentration of 1 g L^{-1}. Reference tests performed with 1 g L^{-1} glucose and blanks (no substrate added) were run as control. All batch tests were performed in duplicate.

2.3. Anaerobic batch tests procedure

Batch tests used to select natural-sourced inocula and to carry out the BHP assays were conducted in 500 mL glass bottles with a working volume of 300 mL under thermophilic temperature. For each bottle, 1.5 mL of nutrient stock solution, i.e. 166 g of $(NH_4)_2HPO_4$, 100 g of KH_2PO_4, 10 g of $MgSO_4{\cdot}7H_2O$, 1 g of NaCl, 1 g of $Na_2MoO_4{\cdot}2H_2O$, 1 g of $CaCl_2{\cdot}2H_2O$, 0.6 g of $FeCl_3{\cdot}6H_2O$ and 1.18 g of $MnSO_4{\cdot}2H_2O$ (Lay et al., 1999), was added to meet the requirement for microbial growth. Initial pH was adjusted to of 5.5 ± 0.2 using NaOH or HCl 1 mol L^{-1} and no buffer solution was added, as indicated by various authors (Liu et al., 2006; Van Ginkel et al., 2005).

All bottles were flushed with nitrogen gas, capped tightly with butyl rubber and incubated at 55 ± 1 °C, until no further bio-hydrogen production was detected (normally within 10 days). For each inoculum/initial substrate concentration tested, the experiment was conducted in duplicate under identical experimental conditions. Batch tests were periodically analyzed for both quantitative and qualitative determination of biogas production. Quantitative production was estimated by withdrawing the extra-pressure gas with syringes of 10–100 mL. The biogas production of blank controls was subtracted from the biogas production of each sample. Hydrogen, methane and carbon dioxide concentrations in biogas were measured by a gas chromatograph (Agilent, Micro GC 3000A) equipped with two thermal conductivity detectors (TCD) and two different columns. H_2 and CH_4 concentrations were measured using a Molesieve/5A Plot column with N_2 as carrier gas at a flow rate of 30 mL/min. CO_2 content in the biogas was analyzed using a different column (Alltech HP-PLOT U) and He as carrier gas at a flow rate of 30 mL min^{-1}. The operational temperature of the injection port was 100 °C, while that of Molesieve/5A and PLOT U columns was maintained at 100 and 55 °C, respectively.

2.4. H_2 productivity in continuously-fed bioreactor

As the best performing organic material found in BHP tests, OFMSW was used to feed a lab-scale continuous flow stirred tank reactor (CSTR) with a working volume of 2.3 L. The feedstock material was mixed with SS (2:3 on wet weight), according the blending procedure used in the anaerobic digestion plant where OFMSW was sampled.

The prepared feeding mixture had a TS content of 126 ± 2 g kg^{-1} and a VS content of 108 ± 3 g kg^{-1} and was intermittently supplied to the reactor every 3 h by peristaltic pump (Masterflex L/S, Cole-Parmer), withdrawing an equal amount of effluent from the reactor. An HRT of 3 days was established according to previous experiences on similar organic materials (Liu et al., 2006), which resulted in an OLR of 36 ± 2 g_{VS} $L_{reactor}^{-1}$ d^{-1}.

As traditional CSTR plants, the digester was continuously stirred by an impeller with a constant speed of 100 rpm to ensure mixing the feedstock and fermentation broth and therefore favoring biogas production. The internal temperature was kept constantly at 55 ± 1 °C by water jacket piping surrounding the reactor.

During the measurement observation period which lasted 35 days, pH and temperature of the fermentative broth were continuously acquired by an electrode probe (InPro 3253/225/pt1000, Mettler–Toledo). A gas flowmeter sensor (ADM 2000, Agilent Technologies) measured with a frequency of 1 Hz the biogas flow at reactor's outlet.

Measured data and actuators control was managed by an industrial PC equipped with an input/output board and a purposely-developed software.

The produced biogas was daily sampled for composition analysis by GC as previously described. The bioreactor start up phase was carried out using the best natural-sourced inoculum selected, sparged for 30 min with N_2 at a flowrate of 100 mL min^{-1}, to remove dissolved O_2 and to establish strict anaerobic conditions. Liquid samples were withdrawn from the fermentation broth every 3 days for chemical analyses.

2.5. Analytical methods

Total solids (TS), volatile solids (VS), COD, total alkalinity (TA), total Kjeldahl nitrogen (TKN) and ammonium nitrogen ($N\text{-}NH_4^+$) were determined according to the standard procedures (APHA, 1998). Total volatile fatty acids (TVFA) and total alkalinity (TA) in the bulk samples were performed on a 5-times-diluted solution of 2.5 g of wet sample filtered to 0.45 nm. TVFAs were determined according to the acid titration method (Lahav et al., 2002). TA was determined in liquid phase by titration with HCl to a pH endpoint of 4.3, as suggested by APHA (1998). Specific VFAs determination (acetic, propionic and *n*-butyric) in the fermentation broth was performed using a different gas chromatograph (Varian, CP-3800) with a capillary column of 25 m × 0.32 mm diameter and flame ionization detector (FID). He at 20 kPa pressure was used as carrier gas, and the temperatures of injector and FID were 220 °C and 240 °C, respectively.

3. Results and discussion

3.1. Naturally-sourced inocula performances

The three inocula obtained from soil (Inocula A, B and C) displayed different behaviors with regard to H_2 production, as reported in Table 2. Inoculum A showed H_2 yields that decreased with increasing substrate concentrations. Inoculum C showed opposite trends (H_2 yields increased with increasing substrate concentrations), while there were no differences in H_2 yields for Inoculum B with different glucose concentrations. In general, no relevant differences were found between Inocula A, B and C in terms of H_2 production efficiencies (Table 2), which in all cases resulted relatively low (0.47–1.35 mol H_2 mol^{-1} glucose, average of 0.91 mol H_2 mol^{-1} glucose), as compared to the best results reported in the literature for mixed microbial cultures (e.g. 2.63 mol H_2 $mol^{-1}_{glucose}$) and for pure/selected/GM ones (e.g. 2.76 mol H_2 $mol^{-1}_{glucose}$) (Fig. 1, Table S1). VFAs concentrations in the aqueous media consistently increased during fermentation, even if no clear dependency on substrate concentrations and H_2 yield could be found. The specific contributions of acetic, *n*-butyric and propionic acids to the total final VFAs contents were very variable, although acetic and *n*-butyric acids were predominant in all trials and propionic acid was rarely detected (Table 2). The predominance of acetate and butyrate was reported by several authors to be ideal for efficient H_2 productions (Antonopoulou et al., 2008). However, in this case, the partial inefficiency of Inocula A, B and C could have been caused by partial incompleteness of acid fermentation (formation of valerate, caproate and other organic acids which were not measured) or due to other metabolic pathways that might have been developed, such as solventogenesis (Hawkes et al., 2002). This led to conclude that, although H_2-producing microbial consortia can be easily obtained from natural sources (soil, in this case), H_2 yields optimization must rely on further developed and specialized microbial consortia, able to follow ideal organic matter fermentation pathways.

3.2. Enhancement of bio-hydrogen yields with acclimated inocula

Inocula D, E and F, fed with glucose at concentration of 3 g glucose L^{-1}, gave higher H_2 yields, as compared to Inocula A, B and C (Table 2). In particular, Inoculum F achieved 2.02 ± 0.05 mol H_2 mol^{-1} glucose (Table 2), i.e. more than double of those obtained with Inocula A, B and C at the same glucose concentration (3 g glucose L^{-1}).

The H_2 yields obtained by Inocula D, E and F were significantly correlated ($r = 0.91$, $P < 0.05$) to the mixing ratio of the soils mixture to the acclimated slurry, used as source of microbial fermentation consortia. At the same time, the H_2 yields were inversely correlated to the TVFA concentrations both at the beginning ($r = 0.90$, $P < 0.05$) and at the end ($r = 0.97$, $P < 0.05$) of the test (Table 2). This led to conclude that TVFA concentrations in culture media were responsible for either direct inhibition of hydrogen-producing bacteria or indirect effects due to pH reduction below optimal levels, as indicated by various authors (Khanal et al., 2004; Van Ginkel et al., 2001). For these reasons, Inoculum F (mixing ratio of 3:1 soil:slurry) resulted in the most performing H_2 production.

For all trials, acetic and *n*-butyric acids were the predominant VFAs produced, in agreement with the work of other researchers

Table 2
Bio-hydrogen yields achieved from glucose by mixed microbial cultures, obtained from natural sources. Volatile fatty acids concentrations measured at the end of the tests.

Inocula	Substrate concentration (g glucose L^{-1})	Hydrogen yield (mol H_2 mol^{-1} glucose)	TVFA at the end of the process ($g_{ACETIC\ ACID}$ L^{-1})	Acetic acid (g L^{-1})	Propionic acid (g L^{-1})	*n*-Butyric acid (g L^{-1})
STEP 1: SOIL INOCULA						
A	1	1.3 ± 0.13	1.23[c]	1.23	–[a]	–[a]
	3	0.89 ± 0.14	2.78	2.32	–[a]	0.67
	5	0.47 ± 0.17	3.83	3.10	0.27	0.75
	7	0.52 ± 0.38	2.32	1.96	–[a]	0.53
B	1	0.88 ± 0.27	0.55	0.55	–[a]	–[a]
	3	0.99 ± 0.18	2.63	1.89	–[a]	1.09
	5	d.l.[b]				
	7	0.72 ± 0.29	6.26	4.62	0.62	1.67
C	1	0.78 ± 0.21	1.07	1.07	–[a]	–[a]
	3	0.85 ± 0.61	3.24	2.84	–[a]	0.59
	5	1.35 ± 0.16	3.72	2.96	–[a]	1.11
	7	1.05 ± 0.28	3.11	1.88	–[a]	1.81
STEP 2: ACCLIMATED INOCULA						
D	3	1.66 ± 0.08	11.41[c]	5.72	0.41	7.86
E	3	1.81 ± 0.02	7.09	4.30	–[a]	4.09
F	3	2.02 ± 0.05	4.26	2.82	–[a]	2.11
F	1	2.8 ± 0.66	3.49	2.39	–[a]	1.61
	5	1.85 ± 0.03	3.85	1.89	–[a]	2.88
	7	1.78 ± 0.08	3.32	2.45	–[a]	1.28

[a] Under detection limit.
[b] d.l. = data lost.
[c] Final concentrations include VFAs contained in the inocula before fermentation and VFAs formed during glucose fermentation.

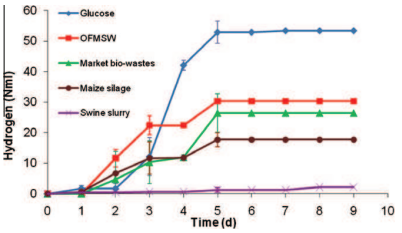

Fig. 2. Batch bio-H_2 potential (BHP) tests applied on four different organic substrates plus glucose (as reference). Cumulative bio-hydrogen production trends (average of duplicates).

(Hawkes et al., 2002), while propionic acid was only detected for Inoculum D. Together with the highest final content of TVFAs, the presence of propionic acid justifies the lowest H_2 yield of Inoculum D, among the tested acclimated inocula. Based on the stoichiometry of glucose fermentation, more hydrogen evolves from acetate-producing than from butyrate-producing fermentation (Lin et al., 2007), while no hydrogen is produced *via* propionic fermentation (Li et al., 2008). Therefore, if the process could be biased towards predominantly acetate production (excepting homoacetogenesis), the yield could be improved (Kyazze et al., 2006). In agreement with this statement, the H_2 yield achieved by using Inocula D, E and F at the same substrate concentration (3 $g_{glucose}\,L^{-1}$) was directly correlated to the increase of the acetate/butyrate ratio ($r = 0.99$, $P < 0.05$).

The identified best performing inoculum (Inoculum F), was applied to test the influence of substrate concentrations on H_2 production. Increased glucose concentrations (1–7 g L^{-1}) caused a progressive decrease in H_2 yield (from 2.8 mol H_2 $mol^{-1}_{glucose}$ to 1.78 mol H_2 $mol^{-1}_{glucose}$) (Table 2). This trend agrees with previous studies that indicated that the increase of substrate concentrations may cause higher levels of inhibitory metabolites and change chemical equilibrium, depressing further hydrogen production (Van Ginkel et al., 2001; Zhang et al., 2003). In this case, TVFAs concentration measured for Inoculum F, even if tested at different glucose concentrations, did not show considerable differences at the end of the trials (Table 2). This revealed that there was a limit on TVFAs concentration, above which further fermentation resulted inhibited or not optimized. For this reason, the lowest substrate concentration used (1 $g_{glucose}\,L^{-1}$) allowed obtaining the best H_2 yield. At the same time, the lowest substrate concentration allowed prevalent production of acetate (high acetate/butyrate ratio of 1.48), i.e. an optimized hydrogen production, as reported by various studies (Van Ginkel et al., 2001; Liu et al., 2006).

In any case, the acclimation strategy (Inoculum F) resulted in harvesting considerably more efficient microbial consortia and in improving H_2 yield, with respect to the soil inocula (Inocula A–C) (Table 2). The best H_2 yield obtained (2.8 ± 0.66 mol H_2 $mol^{-1}_{glucose}$) was comparable to the best results cited in literature for mixed and also for both pure/selected and genetically modified microbial cultures, as shown in Fig. 1 and reported in Table S1.

3.3. BHP tests on selected organic substrates

The BHP tests on four selected organic substrates were performed following the methodology developed in the previous section (Inoculum F, substrate concentration 1 $g_{VS}\,L^{-1}$). As demonstrated, this methodology allows the prompt presence of an efficient microbial community and avoids microbiological or metabolic inhibiting conditions, so that it is ideal for measuring the potential bio-H_2 production of a whatever substrate.

Representative trends of the H_2 production obtained are showed in Fig. 2. The H_2 production reached, in all cases, a plateau after 5 days and the duplicates showed high repeatability. As expected, the control test (fed with glucose) achieved almost the same H_2 production (2.9 ± 0.09 mol H_2 $mol^{-1}_{glucose}$) previously reached at the same substrate concentration. Among the tested biomasses, OFMSW and MBW produced the highest total amount of hydrogen, i.e. 202 ± 3 and 176 ± 2 $NL_{H2}\,kg^{-1}_{VS}$, respectively, while MS reached 118 ± 2 $NL_{H2}\,kg^{-1}_{VS}$ and SS only 14 ± 1 $NL_{H2}\,kg^{-1}_{VS}$ (Table 3).

While SS, as expected, showed low BHP, OFMSW, MBW and MS showed relatively interesting BHP, as compared to various literature results (Lay et al., 2003; Kim et al., 2004).

3.4. Bio-hydrogen productivity in CSTR

Hydrogen production in CSTR fermentation process was analyzed to gain an insight into possible production rates of real scale plant implementations by using as benchmark the potential values obtained in BHP assays. Indeed, these former values are obtained in most optimal environmental and operative conditions allowed. However, a real-scale successful implementation of biohydrogen production by dark fermentation will only rely on continuously-fed co-fermentation of highly concentrated organic mixtures (typically 50–150 $g_{VS}\,L^{-1}$, for CSTR reactors) achieving H_2 yields not too far from those obtained with BHP tests.

The CSTR was inoculated with Inoculum F and the start-up phase lasted till day 5, when stable biogas production and H_2 content in biogas were established (Fig. 3). The process was continuously operated for more than one month with stable production and pH as shown by graphs in Fig. 3. The broth average pH (5.78 ± 0.10) resulted closely stable around the optimum for hydrogen production (Liu et al., 2006; Lin et al., 2008) without any addiction of chemical agents for pH adjustments, which is quite relevant for practical implementation of the process. The average hydrogen content in biogas was of 35 ± 4% and methane was never found in the biogas, likely due to favorable conditions created by the short HRT and the relative acidity of the broth (Table 4).

Table 3
Bio-hydrogen potential (BHP) productions from four substrates studied and glucose (as reference).

Substrate	TS (g kg^{-1})	VS (g kg^{-1})	H_2 produced		
			Total production (Nml H_2)	NL H_2 kg^{-1} w.w.	NL H_2 kg^{-1}_{VS}
Glucose	1000	1000	53.4 ± 0.5 2.90 ± 0.09mol_{H_2} mol^{-1} glucose	356 ± 9	356 ± 9
Maize silage	335	308	17.7 ± 0.2	39.6 ± 0.5	118 ± 2
Swine slurry	30	24	2.2 ± 0.1	0.4 ± 0.02	14 ± 1
OFMSW	270	240	30.3 ± 0.5	54.5 ± 0.9	202 ± 3
Market bio-waste	112	104	26.4 ± 0.3	19.7 ± 0.2	176 ± 2

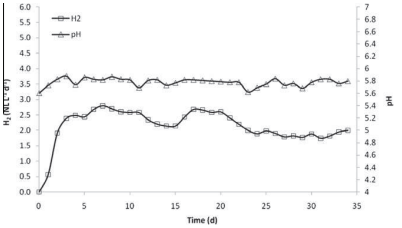

Fig. 3. Trend of bio-hydrogen production and pH in the CSTR system studied.

Table 4
Operative parameters and process performances of the CSTR fermenter, fed with organic fraction of municipal source-separated waste and swine slurry.

	Parameter	Average in the observation period
Substrate	TS (g kg^{-1})	126 ± 2
	VS (g kg^{-1})	108 ± 3
	COD (g kg^{-1})	151 ± 11
	TVFA ($g_{ACETIC\ ACID}$ L^{-1})	4.58 ± 0.2
	TA (g_{CaCO_3} L^{-1})	7.34 ± 0.2
	TVFA/TA	0.62
	TKN (g kg^{-1})	4.4 ± 0.2
	N-NH_4^+ (g kg^{-1})	2.2 ± 0.1
Process	HRT (d)	3
	OLR (g_{VS} L$^{-1}_{reactor}$ d^{-1})	36 ± 2
	Operational average pH	5.78 ± 0.1
	H_2 content in the biogas (%)	35 ± 8
	H_2 production rate (NL H_2 L$^{-1}_{reactor}$ d^{-1})	2.2 ± 0.4
	H_2 yield (NL H_2 kg$^{-1}_{TS\text{-}added}$)	52 ± 4
	H_2 yield (NL H_2 kg$^{-1}_{VS\text{-}added}$)	60 ± 4
Digestate	TS (g kg^{-1})	48 ± 7
	VS (g kg^{-1})	38 ± 5
	COD (g kg^{-1})	58 ± 6
	TVFA ($g_{ACETIC\ ACID}$ L^{-1})	6.45 ± 0.2
	TA (g_{CaCO_3} L^{-1})	5.35 ± 0.6
	TVFA/TA	1.20
	TKN (g kg^{-1})	2.6 ± 0.4
	N-NH_4^+ (g kg^{-1})	1.6 ± 0.3

During the operation the reactor specific production rate of hydrogen resulted 2.2 ± 0.4 NL_{H2} $L^{-1}_{reactor}$ d^{-1} with a yield of 60 ± 4 NL_{H2} $kg^{-1}_{VS\text{-}added}$ (Table 4). These results are comparable with or superior to other results achieved in previous laboratory CSTR tests: for example, Liu et al. (2006) used household solid waste with HRT of 2 days, achieving 43 NL_{H2} kg^{-1}_{VS}. This H_2 production yield represented only the 30% of the BHP measured for OFMSW (202 ± 3 NL_{H2} kg^{-1}_{VS}). This was probably linked to the fact that high substrate concentrations induce high metabolites concentrations, as demonstrated before (Fig. 1): TVFA concentrations were very high in the digestate (Table 4), as compared to the batch tests (Table 2), even if relatively high TA allowed the pH to remain stable (Fig. 3).

Other authors reported very similar results of the CSTR performance with concentrated organic mixtures (Table S2). Perhaps, the CSTR process, which was here intentionally studied because it is actually the most diffused technology in full-scale biogas plants, is not the best solution for achieving optimized dark fermentation and, especially when the process is run with relatively high organic matter concentrations (>100 g_{VS} $kg^{-1}_{wet\ weight}$), is still relatively far from meeting the maximized BHP. As demonstrated by recent works, more satisfactory performances might be reached by using different strategies, taking into account hydrodynamics and reaction kinetics models to improve the bioreactor design (Ding et al., 2010; Wang et al., 2010), although all these experiences were based on low-concentrated and promptly-soluble organic substrates such as glucose and molasses. Other process types, such as leaching bed reactors (Han and Shin, 2004), should be further investigated, for improving dark fermentation reliability as a really applicable technology.

4. Conclusions

It is possible to obtain efficient mixed microbial cultures from natural sources, by proper acclimation to organic substrates. Such types of inoculum, used in batch reactors with proper substrate (1 $g_{glucose}$ L^{-1}) and metabolites concentrations, allowed H_2 yields comparable to pure/selected/GM microbial cultures achievements. This methodology (BHP test), can be applied to organic substrates of possible interest for future applications, to test their potentiality of producing bio-H_2. However, the CSTR fermentation of a concentrated mixture of OFMSW, resulted in around 30% of its BHP, suggesting that further efforts are needed for future applications of dark fermentation in full-scale plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.05.088.

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Supplemental data

Looking for practical tools to achieve next-future applicability of dark fermentation to produce bio-hydrogen from organic materials in Continuously Stirred Tank Reactors.

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Supplemental data contains:

- Table S1 and S2
- Literature referred to tables S1 and S2.

Table S1 – Maximum bio-H2 yields obtained in batch experiments with genetically modified, pure/selected and naturally-sourced inocula in literature and in this study

Source of microorganisms	Operational conditions	Substrate (glucose) concentration (g/L)	Yield (mol H_2/mol glucose)	Reference
Genetically modified				
Enterobacter aerogenes HU-101 (mutant A-1)	37° - pH 6.8	20	0.84	Rachman et al. 1997
Enterobacter aerogenes HU-101	37° - pH 6.8	20	0.83	Rachman et al. 1997

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(mutant HZ-3)				
Enterobacter aerogenes HU-101 (mutant AY-2)	37° - pH 6.8	20	1.17	Rachman et al. 1997
Escherichia coli BW25113 (mutant hyaB hybC hycA fdoG frdC ldhA aceE)	37°	18	1.35	Maeda et al. 2007
Escherichia coli K-12 strain W3110 (mutant SR15, ΔldhA, ΔfrdBC)	37° - pH 6	10.8	1.82	Yoshida et al 2006
Escherichia coli K-12 strain W3110 (mutant SR14 ΔhycA, ΔldhA, ΔfrdBC)	37° - pH 6	10.8	1.87	Yoshida et al 2006
Escherichia coli BL21 (DE3 ΔiscR pAF pYdbK)	37° - pH 7	5	1.88	Akhtar and Jones 2009
Escherichia coli BW535 (mutant JW135 Hyd1-, Hyd2-derivative)	37°	4	1.25	Bisaillon et al. 2006
Escherichia coli BW535 (mutant LJT135, idhA mutant of JW135)	37°	1	1.55	Bisaillon et al. 2006
Escherichia coli BW535 (mutant FJT135, fhlA mutant of JW135)	37°	5	1.4	Bisaillon et al. 2006
Escherichia coli BW535 (mutant DJT135, idhA, fhlA mutant of JW135)	37°	0.3	1.98	Bisaillon et al. 2006
Escherichia coli BW135 (mutant DJT135, Δhya-Km, Δhyb-Km, ΔldhA,	35° - pH 4.5	4.5	0.77	Gosh and Hallenbeck 2010

fhlA-C)

Pure cultures w.t.				
Enterobacter aerogenes HU-101	37° - pH 6.8	20	0.56	Rachman et al. 1997
Escherichia coli K-12 strain W3110	37° - pH 6	10.8	1.08	Yoshida et al 2006
Escherichia coli BW25113	37°	18	0.65	Maeda et al. 2007
Rhodopseudomonas Palustris P4	30° - buffer solution	1	2.76	Oh et al. 2002
Rhodopseudomonas Palustris P4	30° - buffer solution	5	1.3	Oh et al. 2002
Rhodopseudomonas Palustris P4	30° - buffer solution	10	0.98	Oh et al. 2002
Rhodopseudomonas Palustris P4	30° - buffer solution	20	0.7	Oh et al. 2002
Rhodopseudomonas Palustris P4	30° - buffer solution	50	0.66	Oh et al. 2002
Citrobacter sp. Y19	36° - pH 6-7	1	2.49	Oh et al. 2003b
Citrobacter sp. Y19	36° - pH 6-7	5	1.4	Oh et al. 2003b
Citrobacter sp. Y19	36° - pH 6-7	10	1.05	Oh et al. 2003b
Citrobacter sp. Y19	36° - pH 6-7	20	0.7	Oh et al. 2003b
Citrobacter sp. Y19	36° - pH 6-7	50	0.7	Oh et al. 2003b
Clostridium sp. No. 2	36° - pH 6	10	1.99	Taguchi et al. 1994
Enterobacter aerogenes strain HO-39	38° - pH 6-7	10	1	Yokoi et al. 1995
Enterobacter cloacae IIT-BT 08	36° - uncontrolled pH (initial 6.0)	10	2.2	Kumar and Das 2000
Clostridium beijerinckii AM21B	36° - uncontrolled pH	10	2	Taguchi et al. 1992

Citrobacter Freundii	-	7.7	1.29	Kumar and Vatsala 1989
Clostridium pasteurianum	-	7.6	1.5	Brosseau et al. 1982
Citrobacter intermedius	-	7.6	1	Brosseau et al. 1982
Citrobacter intermedius	35° - pH 4.5	22.5	0.35	Brosseau et al. 1982
Citrobacter intermedius	35° - pH 8.5	4.5	1.54	Brosseau et al. 1982
Citrobacter intermedius	35° - pH 8.5	22.5	0.29	Brosseau et al. 1982
Citrobacter intermedius	25° - pH 6.5	4.5	1.34	Brosseau et al. 1982
Citrobacter intermedius	25° - pH 6.5	22.5	0.23	Brosseau et al. 1982
Citrobacter intermedius	45° - pH 6.5	4.5	0.91	Brosseau et al. 1982
Citrobacter intermedius	45° - pH 6.5	22.5	0.23	Brosseau et al. 1982
Citrobacter intermedius	25° - pH 4.5	13.5	0.18	Brosseau et al. 1982
Citrobacter intermedius	25° - pH 8.5	13.5	0.43	Brosseau et al. 1982
Citrobacter intermedius	45° - pH 4.5	13.5	0.29	Brosseau et al. 1982
Citrobacter intermedius	45° - pH 8.5	13.5	0.56	Brosseau et al. 1982
Citrobacter intermedius	35° - pH 6.5	13.5	1.69	Brosseau et al. 1982
Klebsielle oxytoca HP1	35° - initial pH 7.0	9	1.1	Minnan et al. 2005
Klebsielle oxytoca HP1	35° - initial pH 7.0	18	0.36	Minnan et al. 2005
Klebsielle oxytoca HP1	35° - initial pH 7.0	27	0.22	Minnan et al. 2005
Klebsielle oxytoca HP1	35° - initial pH 7.0	36	0.13	Minnan et al. 2005
Ethanoligenens harbinense B49	35° - uncontrolled pH (initial	14.5	2.2	Guo et al. 2009

	6.0) (optimized nutrients concentration)			
Naturally-sourced mixed cultures				
Mixed bacterial cultures from (anaerobic granular sludge from a UASB reactor)	37° - pH 7.5	5	1.46	Davila-Vazquez et al. 2008
Heat-conditioned anaerobic digested sludge	35° - pH 6-7	2	1.4	Kawagoshi et al. 2005
Unconditioned anaerobic digested sludge	35° - pH 6-7	2	1.3	Kawagoshi et al. 2005
Refuse compost with pH conditioning	35° - pH 6-7	2	0.5	Kawagoshi et al. 2005
Kiwi soil heat treated or with pH conditioning	35° - pH 6-7	2	0.5	Kawagoshi et al. 2005
Lake sediment	35° - pH 6-7	2	0.9	Kawagoshi et al. 2005
Dewatered and thickened sludge from a wastewater treatment plant	30° - pH 6.2	3.76	1.17	Salerno et al. 2006
Dewatered and thickened sludge from a wastewater treatment plant	30° - initial pH 6.2 - CO_2 scavenging (KOH)	2	2	Park et al. 2005
Dewatered and thickened sludge from a wastewater treatment plant	30° - initial pH 6.2 - no CO_2 scavenging	2	1.4	Park et al. 2005
Anaerobic sludge from a local municipal sewage treatment plant,	37° - pH 6.0	10	1.75	Zheng and Yu 2005

heat treated				
Centrifugate of digested sewage sludge, heat pretreated	60° - pH 7	10	1.8	Zurawski et al. 2005
Compost	37° - Initial pH 5.5	14.0	1.0	Van ginkel et al. 2001
Compost	37° - Initial pH 5.5	21.0	0.8	Van ginkel et al. 2001
Compost	37° - Initial pH 5.5	28.0	0.9	Van ginkel et al. 2001
Compost	37° - Initial pH 5.5	35.0	0.5	Van ginkel et al. 2001
Compost	37° - Initial pH 5.5	42.0	0.4	Van ginkel et al. 2001
Compost	37° - Initial pH 5.5	1.4	2.2	Van ginkel et al. 2001
Compost	37° - Initial pH 5.5	2.8	1.9	Van ginkel et al. 2001
Compost	37° - Initial pH 5.5	0.5	2.6	Van ginkel et al. 2001
Potato soil	37° - Initial pH 5.5	14.0	0.91	Van ginkel et al. 2001
Potato soil	37° - Initial pH 5.5	21.0	0.67	Van ginkel et al. 2001
Potato soil	37° - Initial pH 5.5	28.0	0.48	Van ginkel et al. 2001
Potato soil	37° - Initial pH 5.5	35.0	0.37	Van ginkel et al. 2001
Potato soil	37° - Initial pH 5.5	42.0	0.29	Van ginkel et al. 2001
Soybean soil	37° - Initial pH 5.5	14.0	0.84	Van ginkel et al. 2001
Soybean soil	37° - Initial pH 5.5	21.0	0.50	Van ginkel et al. 2001
Soybean soil	37° - Initial pH 5.5	28.0	0.46	Van ginkel et al. 2001
Soybean soil	37° - Initial pH 5.5	35.0	0.32	Van ginkel et al. 2001
Soybean soil	37° - Initial pH 5.5	42.0	0.40	Van ginkel et al. 2001

Tomato plants soil, heat treated	26° - pH 6	0.9	0.92	Logan et al. 2002
Palm oil mill effuent (POME) sludge	50° - uncontrolled pH	10.0	0.38	Morimoto et al. 2004
Palm oil mill effuent (POME) sludge	60° - pH 7	10.0	0.43	Morimoto et al. 2004
Sludge compost (from Malaysia)	50° - pH 7	10.0	0.85	Morimoto et al. 2004
Sludge compost (from Malaysia)	60° - pH 7	10.0	0.93	Morimoto et al. 2004
CREST compost from a compost manufacturing plant (Philippines)	50° - pH 7	10.0	0.50	Morimoto et al. 2004
CREST compost from a compost manufacturing plant (Philippines)	60° - pH 7	10.0	0.96	Morimoto et al. 2004
CREST compost from a compost manufacturing plant (Philippines)	60° - pH 7	10.0	1.25	Morimoto et al. 2004
Dewatered anaerobic sludge (heat treated)	25° - 6.2	2.8	0.97	Oh et al. 2003a
Anaerobic sludge at a local cattle manure treatment plant (acid treatment)	35.5° - pH initial 7	21.3	0.90	Cheong and Hansen 2006
Our results				
Mixed culture form soils - Inoculum A	55° - pH uncontrolled (initial 5.5)	1	1.30	This study
Mixed culture form soils - Inoculum A	55° - pH uncontrolled (initial 5.5)	3	0.89	This study
Mixed culture form	55° - pH	5	0.47	This study

soils - Inoculum A	uncontrolled (initial 5.5)			
Mixed culture form soils - Inoculum A	55° - pH uncontrolled (initial 5.5)	7	0.52	This study
Mixed culture form soils - Inoculum B	55° - pH uncontrolled (initial 5.5)	1	0.88	This study
Mixed culture form soils - Inoculum B	55° - pH uncontrolled (initial 5.5)	3	0.99	This study
Mixed culture form soils - Inoculum B	55° - pH uncontrolled (initial 5.5)			This study
Mixed culture form soils - Inoculum B	55° - pH uncontrolled (initial 5.5)	7	0.72	This study
Mixed culture form soils - Inoculum C	55° - pH uncontrolled (initial 5.5)	1	0.78	This study
Mixed culture form soils - Inoculum C	55° - pH uncontrolled (initial 5.5)	3	0.85	This study
Mixed culture form soils - Inoculum C	55° - pH uncontrolled (initial 5.5)	5	1.35	This study
Mixed culture form soils - Inoculum C	55° - pH uncontrolled (initial 5.5)	7	1.05	This study
Mixed culture (Inoculum F)	55° - pH uncontrolled (initial 5.5)	1	2.8	This study - Acclimation strategy
Mixed culture (Inoculum F)	55° - pH uncontrolled (initial 5.5)	3	2.02	This study - Acclimation strategy
Mixed culture (Inoculum F)	55° - pH uncontrolled (initial 5.5)	5	1.85	This study - Acclimation strategy
Mixed culture (Inoculum F)	55° - pH uncontrolled (initial 5.5)	7	1.78	This study - Acclimation strategy

Table S2 – Dark fermentation performances with concentrated organic waste mixtures in Continuously Stirred Tank Reactors (CSTR) at different process conditions, in literature and in this study

Fed subst.	**T**	**Fed subst. concentration**	**HRT**	**OLR**	**Prod. rate LH_2**	**Max. yield LH_2**	**Ref.**
	°C	**g_{VS} L^{-1}**		**g_{VS} L^{-1} d^{-1}**	**L^{-1} d^{-1}**	**kg_{VS}^{-1}**	
OFMSW	55	126	3 d	36	2.2	60	This study
slaughterhouse and food waste	55	28.2	2 d	14.1	0.2	16.5	Karlsson et al., 2008.
food waste	37	40	5 d	8	1	125	Shin and Youn, 2005.
pretreated dairy manures	36	70	32 h	46.5	0.8	31.5	Yan et al., 2010.
pig slurry	70	33.3	1 d	33.3	0.1	4	Thomas et al., 2009.
palm oil mill effluent	55	27.5	2 d	13.8	1	77	Isnazunita et al., 2011.
Beet Sugar Wastewater	35	10	10 h	24	1.1	44.5	Zhu et al., 2009.
Household solid waste	37	75	2 d	37.5	1.6	43	Liu et al., 2006.
cheese whey	36	10	1 d	10	0.5	45	Yang et al., 2007.

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III

Two-stage vs. single-stage fermentation process: comparison of energetic performances and chemical characterization

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Abstract

Two-stage anaerobic digestion (AD) is claimed to be an innovative biological strategy to gain environmental-friendly energy vector (H_2) from waste biomasses and to improve traditional AD process in terms of waste stabilization efficiency and net energy recovery. A two-stage laboratory-scale CSTR digester, fed with a mixture of agricultural and livestock residues, was successfully run for 700 hours and compared to a traditional single-stage reactor. High hydrogen yields (140 Ndm^3 H_2 $kg^{-1}_{VS\text{-}added}$) were reached, with subsequent methane production of 351 Ndm^3 CH_4 $kg^{-1}_{VS\text{-}added}$. Higher CH_4 yields were achieved in single stage reactor (404 Ndm^3 CH_4 $kg^{-1}_{VS\text{-}added}$), therefore almost the same overall energy recovery was produced by the two processes (13-14 kJ $kg^{-1}_{VS\text{-}added}$). Even slightly lower biodegradation efficiencies were shown by the two-stage process, thus partial inhibition of the methanogenic reactor of the two-stage process is assumed. Nevertheless the two-stage produced biogas with high CH_4 content (≈70%), advantageous for lowering the biogas upgrading cost.

Furthermore, this study also propose the GC-MS technique as a novel diagnostic instrument to explore in depth the digestion process

and the variety of biochemical reactions actually occurring into the reactor.

1. Introduction

Anaerobic digestion is one of the effective technologies used to recover energy resources from organic wastes and it is a simple and effective biotechnological mean of reducing and stabilizing organic wastes (Mata-Alvarez et al., 1993; Ueno et al., 2007). This process has been applied to an increasing number of complex feedstock like municipal wastewater sludges, chemical and industry wastewaters and sewage (Demirel and Yenigun, 2002).

Conventional fermentation involves different consortia of bacteria: acidogenic bacteria break down the substrates into mainly H_2, acetic acid and CO_2, while the methanogenic bacteria convert these products to methane gas. A variety of higher organic acids, such as propionic, butyric and lactic, as well as alcohols and ketones, are also commonly formed during the breakdown of the organic substrates by the acidogens, but, in a well operating process, these products are mostly converted to acetic acid and H_2 to be further consumed by methanogenic organisms (Cooney et al., 2007; Van Ginkel et al., 2001; Kraemer and Bagley, 2005).

Digestion process is commonly made in single-stage reactor, but considering the delicate balance between these two groups of microorganisms, which differ in terms of physiology, nutritional needs, growth kinetics and sensitivity to environmental conditions (Demirel and Yenigun 2002), a two-stage approach has been proposed to improve the process. Splitting hydrolysis/acidogenesis and methanogenesis and optimizing each phase, could enhance the overall reaction rate and the biogas yield and make the process control easier (Blonskaja et al., 2003; Liu et al., 2006; Mata-Alvarez et al., 1993).

The two-stage approach has also been used to produce hydrogen and methane separately from each phase with two separate bioreactors in series (Lee et al., 2010; Ting and Lee, 2007; Ueno et al., 2007).

Due to its high energy content (122 kJ/g), hydrogen is a promising, clean and sustainable energy carrier and with its higher gravimetric energy density it can be used in electrochemical (fuel cell) and combustion processes or blended with other fuels such as methane (CH_4) to increase the combustion efficiencies (Venkata Mohan et al., 2009).

However, typically only 15% of the energy from the organic source is obtained from the first stage in the form of H_2 (from 1 to 2 mol of H_2/mol of glucose) and this results in 80–90% of the initial chemical oxygen demand (COD) remaining in the wastewater as volatile organic acids (VFAs) and solvents (Das and Veziroglu, 2001; Kraemer and Bagley, 2005; Van Ginkel and Logan, 2005). The addition of a methanogenic reactor in series after the hydrogen-producing reactor is one alternative promising solution for the exploitation of the remaining 85% of the unused substrate and for concomitant removal of organic pollutants (De Vrije and Claassen, 2005; Koutrouli et al., 2009; Logan, 2004; Ueno et al., 2007).

Some authors reported advantages of the two-stage hydrogen-methane process over the conventional single-stage process. The two-stage process enriches different bacteria in each anaerobic digester (Ghosh and Class, 1978) and this brings to extend processable waste species, in particular for the effective utilization of high water content organic waste, to upgrade percent energy recovery (AIST, 2004), to enhance substrate conversion producing a lower chemical oxygen demand effluent (Azbar and Speece, 2001) and to enhance volume reduction of wastes (Cuetos et al., 2007). The process separation in two-stage could also reduce the overall processing time, because the retention time of the hydrogenogenic operation is relatively shorter than conventional methanogenic reactors (Ueno et al., 2007), and permit to have reduced dimension of

the H_2-reactor if a conventional slow-rate methanogenic process is connected as the second stage (Ueno et al., 2007). Moreover this process could increase the stability of the overall process by controlling the acidification phase in the first digester and hence preventing the overloading and/or the inhibition of the methanogenic population in the second digester (Koutrouli et al., 2009).

On the other hand the two-stage process arouses doubts as it adds to complexity and methanogenic and hydrogenogenic baceria are both liable to inhibition by different factors (pH, organic loading rate, temperature) and could independently lower the whole process performance.

For example if low cost substrates rich in carbohydrates, such as organic wastes/wastewater or agricultural residues, are particularly suitable for fermentative hydrogen production (Benemann, 1996; Han and Shin, 2004; Venkata-Mohan et al. 2009), reaching the highest amount of hydrogen per mole of substrate (de Vrije and Claassen, 2005), the simultaneous build-up of undissociated volatile fatty acids, the favourite substrates for methanogenesis, could deplete the buffering capacity of the medium and decrease the pH, inhibiting microbial growth, hydrogen production and the overall process (Van Ginkel et al., 2001).

In this work the same feeding mixture, made up of market bio-waste and animal slurry, was used to compare a two-stage hydrogen-methane process with the traditional single-stage anaerobic digestion process, both run without pH adjustment.

The performance of the two processes was evaluated not only from the perspectives of total energy recovery and biogas production, but also of organic matter degradation yield, digestate chemical characterization and volatile organic compounds (VOCs) present in the reactors gas phase. The content of soluble intermediates like VFA reflects the changes in the metabolic process involved and may influence the variability of hydrogen and methane production (Venkata-Mohan et al., 2009). For the same purpose a specific

GC/MS approach was adopted to describe the process through the VOCs profile, a large group of anthropogenic (xenobiotic) or biogenic organic compounds. Moreover, as the separation of the acidogenesis and methanogenesis processes is said to negatively affect syntrophic association and to prevent interspecies hydrogen transfer (Reith et al., 2003), a microbial community analysis of microflora of the two different reactors of the two-stage process was performed and compared to that of the single-stage.
To our knowledge such a complete comparison between a two stage and a single-stage fermentation system operated with the same process parameters and without pH adjustment of culture media has not been yet reported.

2. Materials and methods

2.1. Inocula and substrates

Hydrogen-producing inoculum consisted in a digested material of a full-scale biogas plant, treating household source-separated bio-waste and agro-industrial by-products. Before the use the digestate was heat-shocked at 100 ºC for 2 h in order to inactivate hydrogenotrophic bacteria and to harvest anaerobic spore-forming bacteria (Liu et al., 2006; Van Ginkel et al., 2005).
The same digestate, without being heat-shocked, was used as inoculum for the methanogenic stage of both two- and single-stage systems. After inoculation, each reactor was sparged for 30 min at 0.1 L min^{-1} with N_2 to remove dissolved O_2 and to obtain anaerobic conditions.
The feeding substrate was represented by a mixture of swine manure and market bio-waste. Swine manure was collected from 4 different private farms near Milan (Italy) and then filtered through a stainless steel sieve (US Mesh No. 10). Market bio-waste consisted of fruits and vegetables residues was obtained from the municipal fruits and vegetable market of Milan (Italy). Before the use, bio-waste was

shredded by a blender and then stored at -20 °C. Total (TS) and volatile solids (VS) detected for the swine manure were of 10 ± 1 and 8.1 ± 0.5 g L^{-1}, respectively, while raw market bio-waste had a TS and VS content of 133 ± 8 and 99.8 ± 4 g L^{-1}.

Before feeding the reactors the bio-wastes were mixed with swine manure in a 25:75 weight/weight (w/w) ratio, getting a TS content of 39.5 ± 2.5 g kg^{-1} (Tab.1). The average chemical oxygen demands (COD) value of the feeding solution measured over the duration of the experiment was of 86 g_{O2} kg^{-1}.

Table 1 shows the characterization of the input mixture in terms of TS, VS, COD, N-NH_4^+, total nitrogen content (Khielldal method) (TKN) content, pH, total volatile fatty acids (TVFA), alkalinity content (TALK) and their ratio (TVFA $TALK^{-1}$), acetate, propionate and butyrate content, biomethane potential (Schievano et al., 2008), and cumulative biological oxygen demand (OD20) (Schievano et al., 2010) .

2.2. Apparatus and process operation

Three continuous flow stirred tank reactors (CSTR) were used in this study and the reactor designs are reported in Fig. 1. The two-stage process consisted of a 3 L hydrogen-producing reactor with 2 L working volume (R1) and a 18 L reactor with 14.7 L working volume for methane production (R2). Similarly, the single-stage process consisted of a 18 L reactor with 14.7 L working volume (R3). The same feeding mixture was added twice a day both to R1 and R3 after the removal of an equal amount (measured as wet weight) of effluent from the reactors. Peristaltic pumps (Masterflex L/S, Cole-Parmer, Mississauga, ON, Canada) were used to supply intermittently the feeding at the predetermined OLR and HRT and both to transfer effluent from R1 to the methane reactor (R2) and to remove effluents of R2 and R3 to a disposal tank.

Hydraulic retention time (HRT) and the corresponding organic loading rates were of 3, 22 and 25 days, and of 13.3, 2.3 and 1.6 g_{TS} L^{-1} day^{-1}, for the reactor R1, R2 and R3, respectively.

Low HRT for R1 was chosen on the basis of the feeding composition, because market bio-wastes are easily hydrolysable and rich in carbohydrate (Venkata-Mohan et al., 2009), and according to previous experiences about optimized bio-hydrogen production from organic waste materials reported in literature (Ueno et al., 1995, 1996; Tenca et al., 2011). Longer HRT were chosen for R2 and R3 (22 and 25 days, respectively), as methanogenic bacteria have higher growth rates (Conklin et al., 2006).

The overall HRT of two- and single-stage processes were equal (25 d), in order to make them comparable. The three digesters were simultaneously and continuously mixed for 15-seconds every 45-seconds and kept at a temperature of 55 ± 2 °C via water bath through water jackets surrounding the reactors.

During the trial period, the pH in the three reactors was not actively controlled or adjusted and was dependent on the process natural conditions. pH and temperature of the fermentative broth were measured in continuous by three different InPro 3253/225/pt1000 electrodes (Mettler-Toledo international inc.). Gas flow-meters (adm 2000 model, Agilent technologies) were installed in each reactor to record automatically the gas production. Biogas volumes were registered as cumulated every minute and, daily, the average (over 24 h) was reported.

2.3. Analysis

Total and volatile solids (TS and VS), Chemical oxygen demand (COD), total (TKN) and ammonium nitrogen were determined according to Standard Methods (APHA, 1998).

Biogas composition was determined using a gas chromatograph (Agilent, Micro GC 3000A) equipped with two thermal conductivity

detectors (TCD) and two different columns. Hydrogen and methane were analyzed using a Molesieve/5A Plot column with nitrogen as the carrier gas at a flow rate of 30 mL/min. The carbon dioxide content was analysed using a different column (Alltech HP-PLOT U) with helium as the carrier gas at a flow rate of 30 mL/min. The operational temperature of the injection port was 100 °C, while that of Molesieve/5A and PLOT U columns was maintained at 100 and 55 °C, respectively.

The analysis of volatile fatty acids in the fermentation broths were performed using a different gas chromatograph (Varian, CP-3800) with a flame ionization detector (FID) and a capillary column 25 m x 0.32 mm in diameter. Helium at 20 kPa pressure was used as the carrier gas, and the temperatures of injector and FID were 220 °C and 240 °C, respectively.

Gaseous emission produced from the three reactors were caught up into a Nalophan^{TM} bags (3 liters volume) connected to the reactor headspaces. Volatile organic compounds (VOCs) from gas samples were analyzed by SPME/GC-MS. A manual SPME device and divinylbenzene (DVB)/Carboxen/polydimethylsiloxane (PDMS) 50-30 µm fiber - Supelco, Bellefonte, PA, USA) was used. The compounds were adsorbed from the gas samples by exposing the fiber, preconditioned for 3 h at 250°C as suggested by the supplier, in Nalophan bags for 30 min at room temperature. A solution of deuterated p-xylene in methanol was used as internal standard (IS) for quantitative analysis. VOC analysis was performed using an Agilent 5975C Series GC/MSD. Volatiles were separated using a capillary column for VOC (HP 5MS, Agilent Technologies, Santa Clara, CA, United States) of 30 m x 0.25 mm (ID) and a film thickness of 0.25 µm. Carrier gas was helium at a flow rate of 1 ml min^{-1}. VOC were desorbed exposing the fiber in the GC injection port for 600 s at 250 °C. A 0.75 mm i.d. glass liner was used and the injection port was in splitless mode. The temperature program was isothermal for 3 min at 35 °C, raised to 200°C at a rate of 8 °C/ min.

The transfer line to the mass spectrometer was maintained at 250 °C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1294 V and collecting data at a m/z range of 33–300. Compounds were tentatively identified by comparing their mass spectra with those contained in the NIST (USA) 98 library. A semi-quantitative analysis, for all the identified compounds, was performed by direct comparison with the internal standard. Results were expressed as $\mu g\ m^{-3}$.

The biodegradability of the organic matter (OM) contained in the digestates was determined by both short-term and long term biological tests: the specific oxygen uptake rate (SOUR) and the biochemical methane potential (BMP) tests, respectively.

Specific Oxygen Uptake Rate (SOUR test) for all the samples was determined by using a standardized method reported in Schievano et al. (2010). Briefly, the cumulative oxygen demand during 20-hours test (OD_{20}: $g_{O2}\ kg^{-1}_{FM}\ 20h^{-1}$) is measured in a water solution during the microbial respiration in degrading a suspended solid matrix. The microbial respiration works out in standardized moisture conditions, and in maximized conditions of both oxygenation and bacteria-substrate interaction, amplifying the differences among the different samples. This test provides a measure of the short-term biodegradability (putrescibility) of the organic matter.

The Biochemical Methane Potential (BMP test) was performed such as reported by Schievano et al. (2008). In brief, organic matrices were incubated with inoculum at a ratio of 1:2 (substrate:inoculum on a TS basis), for 60 days in batch 100-ml serum bottles under thermophilic conditions. According to our previous approach (Schievano et al., 2008), the test was performed under standardized conditions and the total biogas was reported as parameter to evaluate the organic matter performance under anaerobic condition.

Effluents from the reactors R1, R2 and R3 were sampled four times for a period of one months (one sample/week) during steady state processes (i.e. 700 hours). Steady states were assumed to have been

reached when gas evolution rate and the concentration of H_2 or CH_4 (v/v) in biogas, were constant over 15 days.

3. Results

3.1. Biogas production and energetic performance

The two-stage and single-stage processes were operated for about 3 month, anyway in this work we show a period of 700 h during which, processes showed a steady state .

The biogas production during the hydrogen stage (R1) was of 3.5 L L^{-1} d^{-1} with an hydrogen content in biogas around 45% (volume/volume) (v/v) similar to that reported in literature (Chu et al., 2008), using food wastes to produce biogas. No CH_4 was detected during the trial (Tab. 3). Hydrogen production registered was high (1.59 ± 0.27 L_{H2} $L^{-1}d^{-1}$) but the production was discontinuous showing a great variability i.e., 2.7 L_{H2} $L^{-1}d^{-1}$ as maximum and 0.15 L_{H2} $L^{-1}d^{-1}$ as minimum (Fig. 2). This discontinuity could be correlated to the semi-continuous feeding approach and could be further reduced with continuous management of the process.

The average hydrogen yield reached the value of 140 L_{H2} $kg^{-1}_{VS\ added}$, remarkably higher than the results obtained by other Authors (Liu et al., 2006) using similar wastes, i.e., 43 L_{H2} $kg^{-1}_{VS\ added}$ and 96 L_{H2} $kg^{-1}_{VS\ added}$, respectively.

Biogas production for R2 was of 0.7 L L^{-1} d^{-1} with a methane concentration in the biogas of 68% v/v, higher than that reported for similar digestion processes by Lane (1984). Methane produced corresponded, as average, to 0.48 ± 0.07 L_{CH4} $L^{-1}d^{-1}$. On the other hand the single-stage reactor (R3) produced 1 L L^{-1} d^{-1} of biogas with an average methane percentage of 54.5%, that means a methane production rate of 0.53 ± 0.04 L_{CH4} L^{-1} d^{-1}.

A further comparison between the two processes was performed on an energetic basis through hydrogen and methane conversion to

normalized energy units (MJ $kg^{-1}_{VS\ added}$). The total energy yield was outwardly similar, i.e. 13.10 and 13.54 MJ $kg^{-1}_{VS\ added}$ for the two-stage and the single-stage reactor, respectively. The first-stage influenced just for nearly the 13% of the total energy produced by the two-stage system (Tab. 3).

3.2. Removal efficiency

The AD processes determined important reductions of the TS, VS and COD contents of the feeding mixtures (Table 4). In the two-stage reactor VSs decreased from 854 ± 26 $g_{VS}\ kg^{-1}_{TS}$ (influent) to 750 ± 81 $g_{VS}\ kg^{-1}_{TS}$ (R1 effluent) and further to 605 ± 21 $g_{VS}\ kg^{-1}_{TS}$ (R2 effluent), resulting in a VS reduction after first stage and after the overall process of 25% and 63%, respectively.

The single-stage process, that received the same influent of R1, showed a VS content in the digestate of 610 ± 11 $g_{VS}\ kg^{-1}_{TS}$, that means a VS reduction of 69%, very close to that of the two-stage process.

COD analyses performed on both ingestate and digestates, indicated that 69% and 76% of COD was removed during the two and the one stage process, respectively.

3.3. Digestate characterization

Table 4 reported the characteristics of the digested materials, as average of the observation period. pH and VFA concentrations are two of the main environmental factors that regulate the metabolic pathways of anaerobic digestion (Liu et al., 2006). The feeding pH was sub-alkaline (7.2), but due to the chosen operating parameters and to a stable VFA production during acidogenic phase, pH values for R1 were constantly around 5.5, that was in the optimum range for specific hydrogen production (pH of 5.5-5.7) (Khanal et al., 2004; Liu et al., 2006; Van Ginkel et al., 2001). Due to monomers conversion to hydrogen, CO_2 and volatile fatty acids, R1 digestate

showed, for the whole period, an high total VFAs content (VFA of 3840 ± 745 $mg_{CH3COOH}$ kg^{-1}), more than double than the amount in the fed material (VFA of 1600 ± 115 $mg_{CH3COOH}$ kg^{-1}). In particular, acetic acid was the main VFA specie with more than 2500 mg kg^{-1}, propionate approximately double its concentration while butyrate, which was under detection limit in the fed mixture, showed the highest increase reaching the average concentration of 960 mg kg^{-1} (Tab. 4).

On the contrary, the methanogenic reactors R2 and R3 were characterized by high alkalinity content (5050 and 6480 mg_{CaCO3} kg^{-1}, respectively) and by methanogenic bicarbonate production, which acts to buffer the organic acids and to keep the process pH stably on sub-alkaline vales and in the optimal range for the methanogenic activity (Tab. 5) (Cheong, 2005; Pind et al., 2003).

In R2 the VFAs present in R1 digestate were consumed to produce methane and CO_2, as expected during the methanogenic process, and their total content in R2 digestate was drastically reduced to the amount of 756 ± 410 $mg_{CH3COOH}$ kg^{-1}. In particular, acetate content was always below the theoretical inhibiting limits for methanogenesis (Hill, 1982), and butyrate was not found (Tab. 4).

Differently, R3 digestate showed a further lower total VFAs content (VFA of 75 ± 40 $mg_{CH3COOH}$ kg^{-1}) with acetate as the main VFA and butyrate and propionate under detection limit.

Total VFAs content and TVFA/Total alkalinity ratio are sensitive and diagnostic parameters for system imbalance and R2 and R3 showed values of both parameters compatible with stable methanogenic conditions and below the limits commonly indicated for process inhibition (VFA content over 6000 mg L^{-1} and TVFA $TALK^{-1}$ ratio over 0.4) (Chen et al., 2008; Pind et al., 2003). Notwithstanding, R2 parameters values were constantly higher than R3 values (Table 4).

Finally, the two processes final digestates show similar TKN and ammonia content, this latter under the inhibiting level of 3000 mg

$N\text{-}NH_4^+$ kg^{-1} (Chen et al., 2008). The mineralization of the organic nitrogen led to the increase of the $N\text{-}NH_4^+$/TKN * 100 ratio from the 64% for the fed mixture to over 70% for the digestates of both the processes (Massé et al., 2007).

3.4. Volatile Organic Compounds characterization

The GC-MS characterization of the biogas produced by the three bioreactors is reported in Table 5. R1 showed a net preponderance of carboxylic acids (over 70% of the total VOCs) followed by aromatic compounds and alcohols (Table 5). In particular, VFAs were the 96 ± 2 % of total carboxylic acids, while long chain fatty acids (LCFAs) and other compounds represented the rest (Table 6). Among VFAs, a severe prevalence of hexanoic acid was revealed (64 ± 10% of tVFAs), followed by butyric and acetic acids. Aromatic compounds detected were mainly benzene compounds (more than 50% of the total; cymene was the predominant), plus thiazoles, thiophenes, toluene and furans (Table 5).

R2 showed a wider variety of similarly concentrated VOCs, with a prevalence of ketones (nearly 30% of total VOCs), carboxylic acids (15 ± 5%) and aromatic compounds (14 ± 4%). VFAs represented the large majority of carbossylic compounds (92 ± 3%), again with the prevalence of hexanoic acid (nearly 60% of tVFAs) and the presence of acetic, propionic and butyric acids. Similarly to R, among the aromatic compounds cymene represented more than half of them, followed by naphthalene and phenols (Table 5).

R3 showed VOCs relative content in the biogas similar to R2, except a marked predominance of aromatic compounds (more than 30% of total VOCs), lower concentrations of carbossylic acids and alcohols, and LCFA slightly more concentrated than in R2 (Table 5). Once more, hexanoic acid was the main VFA detected, followed by propionic and butyric acids (acetic acid was undetectable; Table 5).

4. Discussion

The present work demonstrates the feasibility of anaerobic digestion separation in two stages, which allowed the simultaneous production of hydrogen and methane.

Furthermore, the two stage process showed a higher methane content in the biogas than that shown by the parallel single-stage process (Tab. 3). This is probably due to the release of carbon dioxide during the first acidogenic phase, allowing for less carbon dioxide within the methane-rich biogas. This could bring many practical advantages for decreasing gas conditioning requirements of methane (Azbar and Speece, 2001).

The small contribution of the hydrogenesis stage to the total energy yield of the two-stage reactor (13% of the total energy produced) was in agreement with other works which demonstrated that hydrogenesis contributes very little to the total energetic potential, and reported, for organic wastes, 15% maximum recovery as hydrogen (Van Ginkel and Logan, 2005).

From the energetic point of view (energy balance; Table 3) the two systems were quite similar, with the single-stage process that exhibited an energetic yield that was 3.9% higher than that of the two-stage system, which was characterized by a methane production slightly lower than single-stage reactor (R2: 0.48 L_{CH4} L^{-1} d^{-1}; R3: 0.53 L_{CH4} L^{-1} d^{-1}). This was in contrast with what found by other authors which showed a total methane production in a two-stage system 19-21% higher than single-stage process (Liu et al., 2006; Mata-Alvarez et al., 1993).

The different energy recovery between the two systems was supported by the higher COD removal efficiency of the single-stage process (> 6.9% with respect to R1 + R2 reactors) (Table 4). These data lead to the hypothesis that R2 digestate was less degraded and therefore that it could contain an unexploited energetic potential. To prove this hypothesis two biological test (BMP and SOUR tests)

were applied to the digestaes to detect the residual biodegradability of the digestate (SOUR-OD20) and to quantify the unexpressed energetic potential (BMP test) held in the digestates.

The BMP tests performed on R2 and R3 digestates, indicated a higher biogas production for R2 digestate (+ 21%) than R3 digestate, i.e., 1.89 $Ndm^3_{CH4}\ kg^{-1}$ and 1.56 $Ndm^3_{CH4}\ kg^{-1}$, respectively.

The digestates degradability measured under aerobic conditions showed, as expected, a severe reduction of the easily biodegradable fractions contained in the fed organic matter after both one and two stages processes. More interestingly, SOUR test indicated for the R2 digestate a biodegradability of the OM double than that of R3 digestate (117 g $O_2\ kg^{-1}$ TS for R2 versus 64 g $O_2\ kg^{-1}$TS for R3; Table 4). Considering that the OD_{20} measured the biodegradability of easily degradable OM (Schievano et al., 2008), these results agree with the higher TVFAs content found for R2 digestate than the R3 digestate. These results, together with high VFA content (accumulation of product coming from the first stage high-acid content effluent - 4000 $mg_{CH3COOH}\ kg^{-1}$ -) and low pH in R2 reactor seem to indicate a slowing/partial inhibition of the methanogenic process in the two-stage system. The residual energy recovery shown by BMP test (0.066 MJ kg^{-1}_{added}) counterbalances the slightly surplus of energy produced by the single-stage when compared with the two-stage process, such as previously discussed.

In order to better clarify the process condition, we further investigated the process by means of GC-MS technique. As VOCs represents the main products (VFA, ketone and alcohols) of the anaerobic digestion, their investigation can be used to better describe the process and to get a better understanding of it. GC/MS was used to detect the types of VOCs present in gas sample of each reactor, as average of 4 samples/reactor. In R1 and R2 samples 120 different compounds were detected on average, while a statistically relevant lower amount of compound (11 ± 6) was found for R3 (Tab. 5). Despite the difference in the number of detected compounds, their

total amounts in ppbv were similar between the three reactors. Due to carbohydrates and proteins degradation and to lipids anaerobic oxidation (Elefsiniotis and Oldham, 1994; Horiuchi et al., 2002), the 72.6% of the total compounds of R1 were carboxylic acids. On the contrary, in R2 the carboxylic acids are considerably lesser (15%) than in R1, because they were consumed by acetogens and methanogens for methane production. Other headspace R2 compounds were ketones (29%) and aromatics (14. If compared to R2, R3 shows a different distribution of VOCs between the classes and the most abundant classes are aromatic compounds (34%), ketones (26%) and siloxanes (14%).

Table 6 shows the relative composition of the carboxylic acids class found in R1, R2 and R3. Short-chain fatty acids (VFA, from 1 to 6 atoms of carbon) constitute nearly the totality of the carboxylic acids compounds of R1 and R2 (96% and 92% respectively) while in R3 they are just the 15% of the class. The analysis of VFA is of particular interest, as they are important intermediate metabolites of anaerobic digestion and known inhibitors in excess concentrations for degradative microorganisms. Moreover, high concentration of a specific VFA is the result of different parameters like the feedstock composition (Dawson and Glenn, 2001), the HRT and OLR adopted for the fermenter, the operational temperature and pH (Banerjee et al., 1998; Rittman and McCarty, 2001; Wang et al., 2005) and therefore could be indicative for specific metabolic pathway.

In particular, hexanoic acid was the highest amongst other acids found in R1 and R2 samples: this data confirms the study of Levy et al. (1981) who reported that the interruption of anaerobic digestion, producing aliphatic acids instead of methane and CO_2, brings to higher levels of caproic acid and that caproate, ethanol and carbon dioxide are the typical products of cellulose-rich substrates fermentation. R1 gas sample shows a butyrate content less than half of hexanoate and other VFAs below the 5%. Differently in R2 sample was detected just the 5% of butyrate, the 13% of propionate

and the 16% of acetate, the main precursor for methane production via anaerobic digestion (Mountfort and Asher 1978; Lata et al. 2002). On the other hand in R3 hexanoic acid, as other VFAs, were under the detection limits of the analysis.

As for the aromatics, phenol, indole and benzene, with and without substituents, were the most abundant compounds detected (data not shown). Together with benzene, phenol is one of the largely present organic compounds in the environment and it has been found frequently in zootechnical slurries, which are the 75% (w/w) of this study's fed mixture. Phenols are the results of tyrosine metabolic pathway (Jacobs et al., 1981; Spoelstra, 1978) and could be further anaerobically degraded by microorganisms both to VFA, with cyclohexanone, caproic acid and propionic acid production, and to methane (Healy and Young, 1979; Fedorak and Hrudey, 1984; Young and Rivera, 1985). Indole could be found in zootechnical slurries, too (Ramadori and Tandoi, 1993) and is produced in rumen and colon of monogastric by microbic degradation of tryptophan (Yokoyama and Carlson, 1979).

5. Conclusion

A two-stage laboratory-scale CSTR digester was successfully run for 700 hours: hydrogen yields averaged 140 Ndm^3 H_2 $kg^{-1}_{VS\text{-}added}$, followed by methane production in the second stage of 351 Ndm^3 CH_4 $kg^{-1}_{VS\text{-}added.}$ Differently from previous works, no energy surplus (counting the production of both H_2 and CH_4) was produced by the two-stage reactor, since higher CH_4 yields were achieved in single stage reactor (404 Ndm^3 CH_4 $kg^{-1}_{VS\text{-}added}$), therefore producing almost the same amount of overall energy (13-14 kJ $kg^{-1}_{VS\text{-}added}$). However, biological analysis together with deeper process characterization (GC-MS technique), suggest partial inhibition of the methanogenic reactor of the two-stage process. Nevertheless the study support the higher CH_4 content (≈70% on the total biogas volume) produced

through the two-stage reactor, and invite to develop GC-MS technique as specific diagnostic instrument for the process.

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Tables and Figures

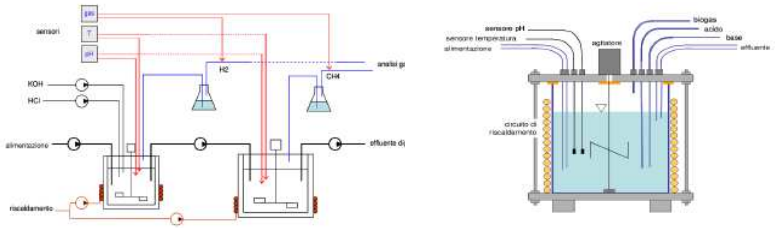

Fig. 1 - a) Schematic diagram of two-stage (a) and single stage (b) process. (1-Hydrogen reactor, 2-Effluent bottle, 3-Methane reactor, 4-Gas meter and counter, 5-Mixer).

Tab. 1 - Characteristics of the input mixture.

TS	$g\ kg^{-1}$	39.5 ± 2.5
VS	$g\ kg^{-1}_{TS}$	854 ± 26
COD	$g_{O2}\ kg^{-1}$	85.9 ± 8.4
TKN	$g\ kg^{-1}$	2.4 ± 1.1
NH_4^+-N	$g\ kg^{-1}$	1.5 ± 0.7
NH_4^+-N TKN^{-1}	%	64 ± 4
pH		7.2 ± 0.1
TVFA	$mg_{CH3COOH}\ kg^{-1}$	1600 ± 115
Acetic acid	$mg\ kg^{-1}$	675 ± 520
Propionic acid	$mg\ kg^{-1}$	172 ± 145
Butirric acid	$mg\ kg^{-1}$	*u.d.l.**
TALK	$mg_{CaCO3}\ kg^{-1}$	4708 ± 10
TVFA $TALK^{-1}$	$kg_{CH3COOH}\ kg^{-1}_{CaCO3}$	0.32
BMP	$N\ dm^3\ CH_4\ kg^{-1}\ TS$	388.1 ± 47.0
	$Ndm^3\ CH_4\ kg^{-1}$	15.53 ± 1.88
OD_{20}	$gO_2\ kg\ C^{-1}_{org}\ h^{-1}$	297.85 ± 16.06
SOUR	$mgO_2\ g\ C^{-1}_{org}$	54.61 ± 8.6

**u.d.l. = under detection limit*

Tab. 2 - Process Parameters.

		Two-stage		Single-stage
		R1	R2	R3
V working	L	2.3	14.7	14.7
V headspace	L	1.1	5	5
Hydraulic Retention Time (HRT)	d	3	22	25
Operational Temperature	℃	55 ± 2	55 ± 2	55 ± 2
TSin	$g\ L^{-1}$	40 ± 1	50 ± 15.7	40 ± 1
Organic Loading Rate (OLR as $_{TS}$)	$g_{TS}\ L^{-1}\ d^{-1}$	13.3	2.3	1.6

Tab. 3 - Biogas, biohydrogen and biomethane production rates and yields for the two-stage and single-stage processes.

		Two-stage		Single-stage
		R1	R2	R3
Volumetric biogas production rate	$Nm^3\ m^{-3}dig\ d^{-1}$	3.5 ± 0.58	0.7 ± 0.1	1.00 ± 0.08
Volumetric hydrogen production rate	$Nm^3_{H2}\ m^3dig\ d^{-1}$	1.59 ± 0.27	0	0
Volumetric methane production rate	$Nm^3_{CH4}\ m^3dig\ d^{-1}$	0	0.48 ± 0.07	0.53 ± 0.04
Hydrogen content in biogas	%	44.9 ± 5.5	0	0
Methane content in biogas	%	0	68.2 ± 1.7	54.5 ± 1.9
Specific hydrogen or methane production on VS basis	$Ndm^3_{H2/CH4}\ kg^{-1}_{VS\ added}$	140.00	324.90	387.90
Volumetric energy production	$kJ\ L^{-1}\ d^{-1}$	19.0 ± 4.9	17.4 ± 2.2	18.5 ± 1.3
Energetic yield	$MJ\ kg^{-1}_{VS\ added}$	1.67 ± 0.37	11.42 ± 1.19	13.54 ± 0.84
Specific total energy production on TS basis	$MJ\ kg^{-1}_{VS\ added}$	13.10 ± 1.24		13.54 ± 0.84

Tab. 4 - Characterization of the digestates of the three reactors observed (R1, R2, R3). Data reported as average of 4 samples during the observation period.

		Two-stage		Single-stage
		R1	R2	R3
TS	$g\ kg^{-1}$	34.0 ± 14.7	21.9 ± 3	17.5 ± 3
VS	$g\ kg^{-1}_{TS}$	750 ± 81	605 ± 21	610 ± 11
COD	$g_{O2}\ kg^{-1}$	58.3 ± 7.4	26.8 ± 3.2	20.9 ± 3.2
TKN	$g\ kg^{-1}$	2.85 ± 0.5	2.2 ± 0.3	1.9 ± 0.2
NH_4^+-N	$g\ kg^{-1}$	1.7 ± 0.3	1.6 ± 0.2	1.4 ± 0.1
NH_4^+-N TKN^{-1}	%	60 ± 4	72 ± 3	72 ± 6
pH		5.52 ± 0.14	7.61 ± 0.06	7.94 ± 0.25
TVFA	$mg_{CH3COOH}\ kg^{-1}$	3840 ± 745	756 ± 410	75 ± 40
Acetic acid	$mg\ kg^{-1}$	2511 ± 345	455 ± 155	53 ± 35
Propionic acid	$mg\ kg^{-1}$	318 ± 140	299 ± 120	*u.d.l.**
Butirric acid	$mg\ kg^{-1}$	958 ± 270	*u.d.l.**	*u.d.l.**
TALK	$mg_{CaCO3}\ kg^{-1}$	4050 ± 980	5050 ± 430	6480 ± 976
TVFA $TALK^{-1}$	$kg_{CH3COOH}\ kg^{-1}_{CaCO3}$	0.95 ± 0.06	0.15 ± 0.03	0.01 ± 0.01
BMP	$Ndm^3\ CH_4\ kg^{-1}\ TS$	318.9 ± 61.5	101.7 ± 8.1	102.8 ± 9.3
	$Ndm^3\ CH_4\ kg^{-1}$	15.27 ± 2.71	1.89 ± 0.05	1.56 ± 0.04
OD_{20}	$gO_2\ kg\ C^{-1}_{org}\ h^{-1}$	270 ± 111	111 ± 40	64 ± 22
SOUR	$mgO_2\ g\ C^{-1}_{org}$	91 ± 33	19 ± 9	10 ± 1

**u.d.l. = under detection limit*

Tab. 5 - Relative area counts (in %) for grouped VOCs found in the reactors headspaces on the total compounds amount in ppbv.

	Two-stage		Single-stage
	R1	R2	R3
Alcohols	4.6 ± 2.4a	5.0 ± 4.6a	0.9 ± 0.9a
Aldehydes	1.1 ± 0.7a	1.9 ± 0.5a	u.d.l.*
Alkanes	3.5 ± 2.4a	7.2 ± 3.7a	7.2 ± 3.2a
Alkenes	0.7 ± 0.5a	2.2 ± 0.8b	1.5 ± 1.4a
Aromatic compounds	7.5 ± 1.1a	13.6 ± 2.9a	33.6 ± 6.7b
Carboxylic acids	72.6 ± 2.9c	15.0 ± 4.9b	7.3 ± 5.4a
Cycloalkanes	0.2 ± 0.1a	1.3 ± 1.1a	2.3 ± 1.9a
Cycloalkenes	0.1 ± 0.1a	1.3 ± 0.7b	u.d.l.*
Esters	1.2 ± 1.0a	u.d.l.*	u.d.l.*
Ethers	0.2 ± 0.2a	1.1 ± 1.1a	u.d.l.*
Halogenated compounds	0.2 ± 0.2a	0.5 ± 0.1a	u.d.l.*
Ketones	1.2 ± 0.2a	29.3 ± 6.5b	25.6 ± 8.1b
Nitrogen compounds	1.1 ± 0.9a	3.9 ± 2.0a	3.9 ± 3.0a
Siloxanes	2.0 ± 1.6a	8.9 ± 0.9ab	14.0 ± 9.5b
Sulphur compounds	0.7 ± 0.7a	1.6 ± 1.9a	u.d.l.*
Terpenes	3.0 ± 0.9a	8.8 ± 3.0b	7.3 ± 2.7ab
Total compounds amount (ppbv)	27167 ± 6332a	23510 ± 10415a	23353 ± 15168a
Total compounds number	123 ± 3b	119 ± 37b	11 ± 6a

[a] number followed by the same letter in the same column are not statistically different (Test Tukey,

** u.d.l. = under detection limit*

Tab. 6 - VFA, with their relative partition into single species, and other carboxylic acids compounds percentage amount on the total carboxylic acids.

	Two-stage		Single-stage
	R1	R2	R3
Volatile Fatty Acids	96 ± 2	92 ± 3	15 ± 3
Formic acid	u.d.l.*	u.d.l.*	u.d.l.*
Acetic acid	5 ± 2	16 ± 8	u.d.l.*
Propionic acid	2 ± 0.5	13 ± 11	u.d.l.*
Butyric acid	25 ± 9	5 ± 5	u.d.l.*
Pentanoic acid	3 ± 3	u.d.l.*	u.d.l.*
Hexanoic acid	64 ± 10	58 ± 26	u.d.l.*
Others	4 ± 2	8 ± 3	85 ± 2

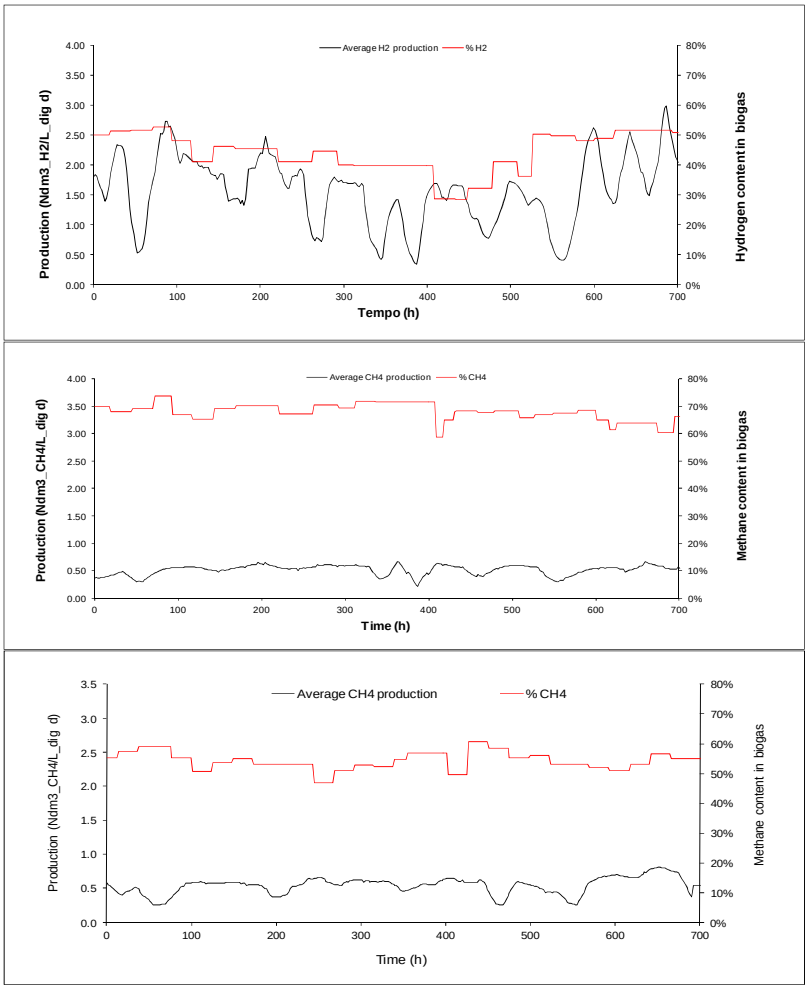

Fig. 2 - Biogas production and its composition in hydrogen (for R1) and methane (for R2 and R3), monitored during the reactors operation (700 h) for R1 (a), R2 (b) and R3 (c).

IV

MEC and Anaerobic Digestion performance comparison with complex industrial wastewater

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Highlights

+ MEC and Anaerobic Digestion comparison

+ Methanol exploitation

+ Molybdenum disulfide as valid alternative catalyst

Abstract

Microbial electrolysis cells (MECs) are a novel bio-technology that can be used to recover energy as hydrogen from organic matter. Through MEC membraneless architecture and unbuffered wastewater use as feeding substrate, methane production was allowed in MEC and organic removal efficiencies and the rate and yield of biogas produced by MEC were compared with simulated Anaerobic Digestion (AD) process. Both the process were fed with an actual industrial wastewater with high methanol content. MEC energy recovery was positive (3.76 and 3.38 kWh/kg $_{TCOD\ removed}$ with platinum and molybdenum disulfide cathode, respectively) and 14-16% higher than that of AD simulation. Also the MEC removal efficiency was high (85-87% TCOD removal, up to 20% higher than

in AD) with complete degradation of methanol, a chemical never reported to be exploited in MEC. MEC emerged to be competitive with the AD process, especially using cheaper alternative catalysts such as molybdenum disulfide (MoS_2).

Keywords

Anaerobic Digestion; MEC; Methanol; Wastewater; Molybdenum disulfide

1. Introduction

Due to depletion of oil reservoirs and climate changes, it is currently of major concern to develop sustainable, environment-friendly and worldwide competitive new technologies for energy production.

The exploitation of biomass, in the case of organic wastes, allow to extract bioenergy and/or biochemicals while treating wastes: in particular, industrial and agricultural wastewaters are ideal candidates because they contain high levels of easily degradable organic material, which results in a net positive energy or economic balance (Angenent et al. 2004).

Several strategies can be used for bioprocessing, including methanogenic anaerobic digestion, biological hydrogen production, ethanol fermentation and fermentation for production of high-value by-products (Angenent et al. 2004, Pham et al. 2006). Recently, bioelectrochemical systems (BESs) for producing hydrogen and electricity have also been developed as a novel biotechnology to harvest energy from soluble biomass. BESs offer the option of electricity production in microbial fuel cells (MFCs) or hydrogen production in microbial electrolysis cells (MECs) (Pham et al. 2006; Thygesen et al. 2010).

Each of the above-mentioned bioprocessing technologies shows complexities and issues that make them advantageous over the other processes in specific fields. Therefore, these bioprocesses are often

combined in parallel with others to maximize the overall energy output (Thygesen et al. 2010) or presented as alternative to the others.

It is well known that anaerobic digestion (AD) is a mature process that allows for the intake of both high and low concentration COD biomass: carbohydrates are particularly well suited, but almost any type of bioavailable substrate is exploitable by AD. Moreover, AD is characterized by high organic removal rates, low energy-input requirements, low sludge production, with final production of energy in the form of methane (Angenent et al. 2004). However, despite AD is a well established technology, it requires meso- to thermophilic temperatures to achieve sufficient turnover and limited methane solubility and it produces methane which needs to be upgraded from a biogas mix, full of undesirable compounds like H_2S, siloxanes, etc.

Among the BESs, both in MFCs and MECs anode respiring bacteria oxidize organic compounds with the anode electrode acting as electron acceptor. In a MEC the electrons produced are later consumed at the cathode by endothermal reduction of H^+ to H_2, thus requiring an addition of power from an external electrical source (Thygesen et al. 2010).

MECs show high hydrogen yields with high efficiency relative to the electrical input (up to over 400%; Lalaurette et al. 2009), which is typically between +0.2 and +0.9 V applied, an amount much less than that used for water electrolysis (>1.6–1.8 V applied) (Call and Logan, 2008). Moreover, MECs have been proved to efficiently convert into hydrogen a wide range of simple organic materials (such as acetic acid, butyric acid, lactic acid, glucose and cellulose), even if few tests have been conducted using complex mixtures of substrates or actual wastewaters (Lalaurette et al. 2009; Wagner et al. 2009; Cusick et al. 2010). While treating the wastewaters and providing energy in the form of hydrogen, this technology can also reduce solids production and sludge handling cost, and can possibly limit

the release of odors (Logan et al. 2008). Due to their similarity with MFC, it is also possible to assume that higher performance could be reached with MEC treating organic matters with readily available soluble COD, also occurring at ambient temperature (25 °C or less) where anaerobic digestion generally fails due to low reaction rates and high solubility of the methane produced (Pham et al. 2006).

On the other hand, the relatively low power density of MECs, their architecture and especially the requirement for expensive noble metals in the electrodes (typically platinum) are the major limitations that make this technology not mature for real-scale applications.

Aiming at minimizing the costs and at scaling up of the technology to real scale, the ion-selective membranes, which are typically used to obtain relative pure cathodic hydrogen gas, could be removed. Anyway different researchers (Call and Logan 2008; Wagner et al. 2009) reported that in membraneless MECs the reduction of ohmic voltage loss in the cell and of a bulk pH gradient in the liquid, is accompanied by the production of appreciable amount of methane gas. Clauwaert and Verstraete (2008) demonstrated that methanogenesis can easily become dominant in membraneless MECs and that the 65 ± 13% of the influent acetate removed was transformed in methane. In addition, methane production in MEC is more abundant with the use of wastewaters as feeding, as they contain endogenous hydrogen-consuming microorganisms, further favored by relatively long operation cycles for slow degradation of complex organic matter (Logan et al. 2008).

Rather than to consider CH_4 as an undesired by-product, methane production in a MEC could be a valuable result. Indeed, MECs methane production may be stronger than hydrogen production (Clauwaert and Verstraete 2008) and compared to anaerobic digesters MECs don't need to use some of the produced energy for heating the reactor to the temperatures needed for efficient methanogenesis (Logan et al. 2008). Therefore, it must be evaluated if the process performance and the value of the gas produced can

compensate for the electrical energy requirements and the more complex design of MECs compared to ADs.

In the present study instead of searching for strategies to inhibit the methanogenesis in membraneless MECs (Call and Logan 2008; Rozendal et al. 2008), their biogas, and especially methane, production and wastewater treatment efficiency were monitored. Process performances during consecutive fed-batch cycles were compared in membraneless MECs operated in presence of an external applied cell voltage and with no voltage applied. The latter condition intended to simulate an anaerobic digestion process.

Furthermore, as most MEC researches have been done using Platinum as catalyst, which accounts for the greatest percentage of the cost of the MEC and can also be negatively affected by components often present in waste streams (Jeremiasse 2010), alternative catalysts are needed, especially for minimizing real scale application cost. Two alternatives (stainless steel and MoS_2) have been used and compared with Pt performance in this study.

Finally, high-COD industrial wastewater, with a significant content of methanol, was used as feeding substrate. Methanol exploitation in MEC has never been previously reported.

2. Materials and methods

2.1 Wastewater

Industrial wastewater was collected from the wastewater treatment system at Allentown, USA. Samples were placed on ice and shipped overnight to the laboratory and stored at 4° C. Wastewater served both as inoculum and substrate in all experiments and its full characterization is shown in Table 1.

2.2 Reactors construction and operation

A common practice for enriching a bacterial community in a MEC is to operate a MFC for several fed-batch cycles and then transfer the

anode into a MEC. This procedure ensures biofilm formation on the anode and preselects an exoelectrogenic community for subsequent MEC operation (Logan et al. 2008). To this aim, six single-chamber MFCs were built, having a cylindrical chamber 4 cm long by 3 cm in diameter (empty volume = 28 mL) and an anaerobic culture tube glued to fit on top of the reactor (1.6 cm inner diameter and 6 cm length; 12 mL capacity). The adopted anodes were heat-pretreated graphite fiber brush electrodes (PANEX 33 160K, Gordon Brush, OD = 2.5 cm, L = 2.5 cm), while cathodes were flat carbon cloth (Type B-1B, E-TEK, 3.8 cm diameter) added with a Pt catalyst (10% Pt/C) on the anode-facing side of the electrode (Call and Logan 2008). Cathodes used in MFCs were also treated to have four diffusion layers (PTFE) applied to their airfacing side (Cheng et al. 2006).

The six reactors were operated as MFCs for two months (details given in supporting information) in order to enrich biofilm on the anodes surface, with consequent current production, directly using the wastewater without any dilution or amendment.

After evidence of relatively stable exoelectrogenic activity, the reactors were converted to operate as MECs as described by Call and Logan (2008), both by replacing the cathodes and by covering them with a plate to exclude air and eliminating the oxygen reduction at the cathode.

Three different cathode types were used, in order to compare the performance of different catalysts: Platinum (Pt), Molybdenum disulfide (MoS_2) and stainless steel (SS). All MEC cathodes had a total surface area of 12 cm2, with only 7 cm2 exposed to the solution, and no diffusion layers.

Carbon cloth cathodes with a Pt catalyst were constructed using a mixture 10/90 of platinum powder and carbon black (E-TEK, C1-10, 10 wt.% Pt on Vulcan XC-72).

Carbon cloth cathodes with MoS_2 catalyst were prepared using a MoS_2 powder (Aldrich, 99%, particle size < 2 mm), mixed with 5

mg/cm^2 carbon black (Cabot, VULCAN XC-72R, > 99%) at mass loading ratios of 33.3% and with 50 µL/cm^2 of a 2:1 volume solution of Nafion polymer (Aldrich, 5 wt%) and iso-propanol. After vortexing the mixture for 15 s at 3200 rpm (VWR Vortex Mixer) this was applied just to the solution side of the carbon cloth (E-TEK, B-1/B/30WP, 30% by weight PTFE Wet-Proofed).

For SS cathodes, flat sheets of type 304 stainless steel (Trinity Brand Industries, Inc.) were sanded smooth with silicon carbide sand paper, then ultrasonically washed in deionized water and rinsed with acetone, with a final rinse in deionized water and drying overnight (>12 h) before testing.

A positive voltage (E_{ap}) of 0.7 V was applied to the MECs by connecting the negative pole of an external power supply (model 3646A; Circuit specialists, Inc.) to a resistor (10Ω) and then to the cathode, and the positive pole to the anode. Instead, for anaerobic digestion simulation, no voltage was applied (Open Circuit Voltage) to the cells (OCVCs). When the complete gas production cycle ended, as indicated by zero gas production rate for one hour or more, both the MECs and the OCVCs were drained, refilled with fresh substrate, and flushed with ultra high-purity nitrogen gas (99.998%) for 15 minutes.

All the tests were run in fed batch mode with duplicate reactors at 30° C in a constant temperature room.

2.3. Measurements and chemical analyses

Reactor voltage was measured across an external resistor (MFC: Rex = 1 kΩ, MEC: Rex = 10 Ω) every 20 min using a multimeter (2700; Keithley, United States) connected to a personal computer. Current and power generation were calculated using I = E/R and P = IE respectively, where I (A) is the current, P (W) the power, E (V) the voltage and R (Ω) the resistance.

During each fed-batch cycle the volume of gas produced was recorded using a respirometer (AER-200; Challenge Environmental) and the evolved gas was collected in air-tight gas bags (0.2 L capacity; Cali-5-Bond, Calibrated Instruments, Inc.). Gas from the gas bag and the reactor headspace was sampled using a gas-tight syringe (200 µL injection volume) and analyzed using gas chromatography (Models 310 & 8610B; SRI Instruments, Torrence, CA).

The influent and the effluent of the MECs were characterized for each batch cycle. The concentrations of solvents, alcohols, and organic acids (acetone, methanol, ethanol, propanol, butanol, acetate, propionate, and butyrate) were measured by gas chromatography (Varian Star 3400) with injector and flame ionization detector temperatures of 250 °C. Total and soluble COD were quantified through HACH method 8000 (HACH COD system, HACH Company, Loveland, CO). Probes were used to measure pH (Mettler Toledo Seven Multi; Model: pH; S/N: 290843) and conductivity (Mettler Toledo Seven Multi; Model: Cond.; S/N: 291048).

2.4. Calculations

Biogas Production Rate

The volumetric production rate, Q (m^3 gas/m^3reactor d), of both H_2, CH_4 and CO_2 was calculated based on the measured specific gas produced normalized to the reactor volume.

TCOD Removal

The ability of the process to be a feasible treatment technology was expressed both as the total COD removal (% of the TCOD of the feeding wastewater which has been removed at the end of the batch cycle) and as the TCOD removal rate, r TCOD (kg $_{TCOD\ removed}$/m^3reactor d).

Hydrogen and Methane Yield

Being the wastewater used for the experiments a complex source of organic matter, the yield (Y) of hydrogen and methane was expressed on the basis of the COD removal (Nm^3 gas/kg $_{TCOD\ removed}$) and calculated as

$$Y_{gas} = \frac{V_{gas}}{V_r(COD_i - COD_e)}$$

where V_{gas} is the total volume of a specific gas, hydrogen or methane, produced, V_r the reactor volume and COD_i and COD_e the COD concentrations of the wastewater at the beginning and end of the batch test.

COD removal was calculated as the average from two to five batch tests.

Energy Recovery

Energy yield in the MEC or in OCVC was calculated as energy production per unit of reactor volume (kWh/m^3) normalized to COD removal (kg TCOD/m^3).

Energy recovered as gas was determined by multiplying the moles of hydrogen and methane produced by their lower heat of combustion (-286 and -891 kJ/mol respectively) and converting the value to express the yield as kWh/kg $_{TCOD\ removed}$.

As for the MECs, the net energy recovery (kWh/ kg $_{TCOD\ removed}$) of the process was calculated by subtracting the supplemental energy required and added to overcome the potential for hydrogen evolution from the value of energy produced as gas during each MEC batch-cycle. The energy required by MEC system (W_{ap}) is obtained by converting the recorded voltage added by the power source (E_{ap}) taken over time intervals Δt = 20 min for n intervals as followed

$$W_{ap} = \sum_{1}^{n} (I)(E_{ap})\Delta t \left(\frac{1\ kWh}{1000\ Wh}\right)$$

3. Results

3.1 Reactors performance in MFC mode

Four representative cycles of current generation for four cells later converted to MEC are shown in Fig. S.1. Maximal voltage and current density reached were 420 mV (1 kΩ) and 0.59 A/m^2, respectively, similar to the results of other studies with actual wastewaters; for instance winery wastewater with high TCOD (2200 ± 510 mg/L) produced 441 ± 17 mV (1 kΩ) (Cusick et al., 2010). This shows that the wastewater provided a good source of exoelectrogenic bacteria and that their enrichment on the anode was achieved.

During the experiments all the cells showed a similar gradual decrease both for the batch cycle time and the maximal current density reached, decreasing from 6 to 4 days and from 0.60 to 0.46 A/m^2, respectively (Fig. S.1). This, despite the coulombic efficiency remained stably low (7 ± 2%), but within a range typical for MFC treating wastewaters (from 5 to 20%; Min and Logan, 2004; Heilmann and Logan, 2006; Min et al. 2005), as well as the TCOD removal resulted stably high (90%). Again, both the batch cycle time and the TCOD removal are similar to those achieved by Cusick et al. (2010) for winery wastewater, who reported a COD removal of 83 ± 10% in 6 days.

Assuming that the double-peak profile in the current production (Fig. S.1) is indicating a different kinetics in the degradation of different organic fractions, both TCOD removal and VFAs and alcohols content in the liquid solution were analyzed after the end of the first peak of current production (≈ 2.5 days) and at the end of the cycle (Tab. S.1). Anyway, the more abundant compounds in the wastewater, i.e. methanol and acetate, 1.54 and 0.18 g/L respectively, were equally degraded after the first current peak (78% and 75%, respectively) and totally degraded at the end of the cycle (Tab. S.1).

3.2 MEC performance and comparison with OCV

MEC duplicates exhibited reproducible results throughout the experiments in terms of both current generation and biogas production (Fig. S2).

At an applied voltage (E_{ap}) of + 0.7 V, the amount of electrical current produced in MEC resulted to depend on the cathode type: Pt reached the maximal current density of 2.07 A/m^2, higher than MoS_2 and SS catalysts, with 1.4 and 0.95 A/m^2, respectively. Among the MECs, Pt-MEC produced also biogas at the highest average flow rate (1.71 m^3 gas/m^3reactor d), with the highest average H_2 content (32 ± 4%). MoS_2-MEC and SS-MEC had lower performances both in terms of flow rate and of hydrogen content (MoS_2: 1.18 m^3 gas/m^3reactor d, 25 ± 5 % H_2; SS: 0.83 m^3 gas/m^3reactor d, 16 ± 1 % H_2) (Fig. 1). Methane was always found in the biogas, with high relative amount of 55 ± 4 %, 62 ± 3 % and 70 ± 2 % for Pt, MoS_2 and SS catalysts, respectively.

Coulombic Efficiency is low even if compared to other studies with actual wastewaters (Ditzig et al. 2007; Wagner et al. 2009; Cusick et al. 2010): Pt showed the same CE as in MFC mode (7 ± 1%), while MoS_2 and SS reached minimal higher values of 10% and 12.5%, respectively.

Finally, Pt had the shorter cycle time (31 hours), while MECs with MoS_2 and SS as catalysts showed similar and longer fed-batch cycle time (37.5 and 45.5 hours, respectively).

Comparing the biogas production in MEC with that in OCV, differences both for the total amount of biogas produced, production flow rate and gas composition were detected. According to the data averaged over different batch cycles, biogas produced by MEC represented 156%, 155% and 116% of the total amount of biogas produced in OCV with the same catalysts, Pt, MoS_2 and SS respectively.

Considering the production rate (Q), both Pt-MEC and MoS_2-MEC had higher flow rate than their corresponding OCV, while due to its longer batch cycle time SS-MEC had a lower Q than without added voltage (Fig. 1). Lower amounts of hydrogen (Pt: 26 ± 3%; MoS_2: 21 ± 6%; SS: 14 ± 1%) and higher of methane (Pt: 65 ± 3%; MoS_2: 69 ± 7%; SS: 74 ± 1%) were found in OCV biogas (Fig. 1).

The maximal hydrogen yield for this wastewater was obtained by Pt-MEC (0.174 ± 0.001Nm^3 H_2/kg $_{TCOD\ removed}$), while lower yields were achieved with MoS_2 and SS as catalysts (0.117 ± 0.001 and 0.075 ± 0.002 Nm^3 H_2/kg $_{TCOD\ removed}$, respectively) (Fig. 2). Previous MEC tests using domestic wastewater in a two-chamber MEC (Ditzig et al. 2007) resulted in hydrogen yields similar to those obtained here. Both the biogas yield and the hydrogen yield were higher in MEC than in OCV, in particular with Pt as the catalyst (Fig. 2).

Methane yield were higher than hydrogen yield and in particular they were again higher in MEC than in OCV. MEC showed similar methane yields between the different catalysts and a range between 0.286 Nm^3 CH_4/kg $_{TCOD\ removed}$ (SS-MEC) and 0.300 Nm^3 CH_4/kg $_{TCOD\ removed}$ (Pt-MEC) (Fig. 2). The methane yields in OCVCs were lower, with 0.260 ± 0.002, 0.245 ± 0.002, 0.277 ± 0.003 Nm^3 CH_4/kg $_{TCOD\ removed}$ for Pt-MEC, MoS_2-MEC and SS-MEC respectively (Fig. 2).

More interestingly, Fig. 3 shows the overall net energy balance of the processes, expressed as kWh/kg $_{TCOD\ removed}$, considering the amount of energy in the biogas produced (CH_4 + H_2) per kg COD removed. Apart for similar energy recovery with SS, both Pt and MoS_2 catalysts reached higher energy recovery in MEC than in OCV. Pt-MEC got the highest recovery of 3.758 ± 0.003 kWh/kg $_{TCOD\ removed}$, while lower performances were reached in OCV (3.236 ± 0.003 kWh/kg $_{TCOD\ removed}$); with MoS_2 at the cathode 3.379 ± 0.003 and 2.971 ± 0.003 kWh/$_{kg\ TCOD\ removed}$ were respectively achieved in MEC and OCV.

3.3 Treatment efficiency

Consistent and effective treatment of the wastewater was achieved both in OCV and with addition of external voltage of +0.7 V (MEC). TCOD removal efficiency in MEC was always over 85%, with lower performance for SS cathode and higher (89%) for Pt (Fig. 4, Tab. 2) and with results similar to those obtained during the preliminary study in MFC. Considering the time of the batch cycle (31 h, 37.5 h, 47.5 h for Pt, MoS_2 and SS cathodes, respectively) and the relatively high amount of TCOD in the influent (4070 ± 180 mg/L) with high content of methanol (1540 ± 50 mg/L) these removals can be considered quite encouraging positive results.

Ditzig et al. (2007) showed an overall higher COD removal of 95 ± 2% with MEC treating domestic wastewater, which however had a lower initial COD, between 204 and 481 mg/L.

Summarily comparing the MECs with OCVCs, the latter always showed a lower TCOD removal efficiency (between 74% and 81%, Fig. 4, Tab. 2) than MECs. OCVCs lower performances were especially observed with Pt and MoS_2 cathodes. On the contrary, if we consider the removal rate of TCOD (r TCOD, Fig. 4), MEC achieved an higher rate than OCV just with MoS_2 as catalyst (2.26 and 1.87 kg TCOD removed/m3reactor d for MoS_2-MEC and MoS_2-OCV, respectively). However, the overall higher r TCOD was reached by Pt-MEC (2.80 kg TCOD removed/m3reactor d) and Pt-OCV (3.17 kg TCOD removed/m^3reactor d) (Fig. 4). Furthermore, the soluble part of COD (SCOD) is high and similar between the two processes (MEC and OCV) and the different catalysts, being comprised between 78% and 87% of the total COD (Tab. 2).

Tab. 2. shows the content of VFAs and alcohols in the liquid solution of MEC and OCVC for all the cathode types. All the MECs showed just traces of acetate (< 30 mg/L) within the liquid solution and a complete consumption of the methanol present in the feeding, while the OCVCs had a more complex profile with unexpected high

amount of acetate (always higher than 130 mg/L) and uneven detection of methanol, acetone, propionate and butyrate.

4. Discussion

Like previously demonstrated by other researchers (Clauwaert and Verstraete, 2008), persistent methanogenesis was obtained in this study with the use of single chamber MECs, which have a simple reactor design (no membrane) and have been fed with a raw actual wastewater, with high methanol content and likely rich in complex anaerobic microflora.

Moreover, the relatively long cycle time (Fig. S.2) required both for a more complete degradation of the complex high-TCOD organic substrate, and for avoiding kinetic roadblocks from hydrolysis and fermentation in the MEC (Lee et al. 2009), probably resulted in favouring a well established methanogenic community in the biofilm on the electrodes. Therefore, even if this study didn't consider any microbial community characterization, it is presumable the simultaneous presence and activity of fermentative methanogenic bacteria and exoelectrogenes, as hydrolysis and fermentation are both preliminary steps necessary for the utilization of a complex wastewater by exoelectrogenes.

Ditzig et al. (2007) already stated that, compared to the acetate or non fermentable pure compounds, the use of complex wastewaters could bring to a low efficiency of hydrogen recovery (H_2 consumed by other microorganisms) and to a low electron recovery (failure to convert organic matter to current). With domestic wastewater (Ditzig et al. 2007) maximum CE of 26% was reached, compared to CEs of 78% and 92% in previous MEC studies with acetate (Liu et al. 2005; Rozendal et al. 2006). Therefore, in the present study a large percentage of the electrons coming from the COD removed was probably not successfully transferred into current and instead used

for cell growth or for anaerobic digestion process, as indicated by low CEs (7-12.5%) and by the amount of methane found in the cells.
However, the production of biogas with high relative content of methane (always > 55%) gives opportunity for this technology to be compared with classical anaerobic digestion process. Indeed, the present study showed that both Pt and MoS_2 as catalysts achieved higher biogas production rate and biogas yield in MEC than in OCV.
At the temperature of 30 °C, maximal average methane production rate of 0.9 m^3 CH_4 / m^3 reactor day was reached by Pt-MEC while with MoS_2 at the cathode the CH_4 production rate increased from 0.51 in OCV to 0.73 m^3 CH_4/m^3 reactor day in MEC, a rate similar to that shown by Clauwaert and Verstraete (2008) with graphite granules at the cathode.
Hydrogen was found in biogas together with methane, but it was generated at low rates, if compared to other studies with both pure compounds in buffered environment and wastewaters (Cheng and Logan 2007, Call and Logan 2008, Lalaurette et al. 2009, Wagner et al. 2009). Hydrogen production rate was higher in MEC than in OCV and the maximal rate in MEC was achieved with Pt catalyst (0.54 ± 0.08 m^3 H_2/m^3 reactor day).
However it is possible to assume that the higher relative amount of hydrogen produced in MEC (up to 32% of the biogas, v/v, for Pt-MEC) could have been even higher, because an undefined additional amount of H_2 electrochemically produced by exoelectrogenes could have been consumed by hydrogenotrophic bacteria to produce methane, or other reduced compounds as acetate (Thygesen et al. 2010). Tartakovsky et al. (2008) gave evidence that hydrogenotrophic methanogens converted up to 50% of the H_2 produced at the cathode into methane in a continuous-flow MEC.
In order to be really competitive, MEC needs to reach the anaerobic digestion cost-effectiveness level and for this purpose the net amount of energy extracted was calculated, since the energy content in the biogas must offset the energetic cost of the electrical energy

(i.e. applied voltage) consumed in the MEC. MEC energy recovery, considering both the methane and the hydrogen gas produced, was positive (3.76 and 3.38 kWh/kg $_{TCOD\ removed}$ for Pt- and MoS_2-respectively) and 14 - 16 % higher than that of OCV.

As previously stated, the capital cost for the electrodes is still a big obstacle for an easy application of MECs to a large-scale facility, and Tokash and Logan (2011) recently demonstrated that MoS_2 composite cathodes perform similarly to Pt cathodes in terms of current densities, hydrogen production rates and COD removal in MEC fed with simple pure substrate (sodium acetate) and that 10 wt.% platinum composite cathodes are about five times more expensive than similarly constructed composite MoS_2 cathodes. The good energy recovery and overall performance of MoS_2-MEC reached in this study demonstrated that also using wastewaters MoS_2 is a valid alternative to Pt as catalyst, able to achieved the same increase in performances, compared to its corresponding OCVC, obtained with Pt.

Even from the organic removal efficiency standpoint, not only microbial electrolysis system may be a promising alternative to AD (higher TCOD removal) but again the use of MoS_2 catalyst achieved a TCOD removal rate in MEC 20% higher than in OCV.

The study of Ditzig et al. (2007) assumed that the initial COD was affecting the reactor performances, which increased with wastewater strength, suggesting that wastewaters with high organic matter concentration could be useful in immediate applications of bioelectrochemical systems. Here COD-rich wastewater was efficiently treated in MEC (between 85 and 87 % TCOD removed), showing higher performances than previous results with actual wastewaters. Wagner et al. (2009) obtained swine wastewater COD reduction up to 72 ± 4%; Cusick et al. (2010) a TCOD removal of 58 ± 3% and 47 ± 3% with domestic and winery wastewater, respectively. But this study also demonstrates that MEC system has higher organic removal than anaerobic digestion process, simulated

in OCV, therefore gaining another advantage in the comparison between the two processes, since higher TCOD removal means reduction of the effluent waste handling cost.

In MFC the presence of other organisms (mixed culture) than those that can transfer electrons to the anode (pure culture) has been shown to benefit the performance, generating a current six fold higher than that produced by the pure culture (Angenent et al. 2004) and also increasing the COD removal. Similarly, the biological process here established could have been improved by the interaction between the exoelectrogenes bacteria and the hydrogenotrophic methanogens within the thick biofilm on the anode (Lee et al. 2009), increasing the already high TCOD removal achieved in OCV (up to 81% with stainless steel as catalyst). Moreover, also the removal of the high amount of methanol, never before demonstrated in MEC, could have profit by this cooperation. In this specific case of study, the feeding wastewater had an high amount of methanol, which, as an important material of chemical plants, often occurs in chemical wastewaters, even in high concentration and contributed here to the 55% of the total COD of the wastewater (Tab. 1). Previous studies evaluated the exploitation of methanol both in a conventional fuel cell (Logan 2004) and in anaerobic degradation, achieving high removal performance especially in upflow anaerobic sludge blanket reactors (Chen et al., 2000; Paris and Blondeau, 1999; Woods et al., 1989). The 99% of methanol contained in the condensate from a paper mill was biodegraded in a UASB by Park and Park (2003).

In this study, methanol was completely degraded both in MEC and OCVC, except for traces below 12 mg/L in Pt- and MoS_2-OCVC (Tab. 2).

At a pH close to 7.0, i.e. that of this experimentation, methanol could either be directly converted to methane or through formation of acetate or through a combination of both (Bhatti et al. 1996).

$$4CH_3OH \rightarrow 3CH_4 + H_2O + HCO_3 + H^+ \quad (1)$$

Generally, the former is the dominant reaction while the acetate-producing relies on the existence of CO2 or HCO3- and trace elements (Gonzalez-Gil et al. 1999; Weijma and Stams 2001).

Here, the simultaneous absence of methanol in the ffluent of the processes and the increased content of acetate recorded in OCV seem to suggest that the conversion of methanol to acetate via acetoclastic methanogens, expressed as follows

$$4CH_3OH + 2HCO_3 \rightarrow 3CH_3COO + H^+ + 4H_2O \quad (2)$$

is prevailing in this study.

The difference in the acetate content at the end of the process between MEC and OCVC could be connected with the higher TCOD removal and the higher amount of biogas produced in MEC, assuming that additional acetate from methanol fermentation has been efficiently used by the exoelectrogenic bacteria, favored by the added voltage.

Further work on microbial communities characterization in these systems could clarify better the interrelationships between the bacteria involved in the degradation of such a type of industrial, high-methanol content wastewater.

5. Conclusions

A comparison between MEC and Anaerobic Digestion, simulated without adding voltage to a MEC system, was made, regarding energy balance and organic removal efficiency of the two processes.

It is known that MEC is not a technology ready for real-scale application like AD and that its use for wastewater treatment will depend on different factors (the cost of the materials, which for large-scale treatment is yet to known, and especially of the catalysts, the amount of energy needed, etc.).

However substantial methane production was found in single chamber MEC, and with both platinum and molybdenum sulfide at

the cathode higher energy recovery and TCOD removal than in anaerobic fermentation was reached.
Therefore MoS_2 proved to be a valid alternative to Pt at the cathode, much more affordable for pilot- or real-scale appliances. Moreover the MEC technology gave evidence to be a competitive method for efficiently treating unbuffered, raw wastewaters, exploiting the joined different skills of fermentative bacteria and exoelectrogenes to efficiently treat complex wastewaters and specific contaminants, such as methanol in this study.
In order to enhance the advantages toward the classical anaerobic digestion process and to strengthen its application niche, further studies and comparison could be made with MEC treating low concentration COD substrates and at low temperatures (10-20 °C), i.e., where AD does not function well (Pham et al. 2006). Also, as proposed by Logan et al. (2008), research is needed on whether MEC systems will be capable of stand-alone operation or if aerobic effluent polishing must be coupled, as often required by AD.
Alternatively, as proposed by Zeeman et al. (2008), methane-producing MECs could be used in combination with conventional anaerobic digestion as a way to remove residual fatty acid and sulfides.

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Tables and Figures

Tab.1 - Wastewater characterization.

Parameters		
pH		6.68 ± 0.30
Conductivity	mS/cm	2.04 ± 0.02
TCOD	mg/L	4070 ± 180
SCOD	mg/L	3810 ± 160
BOD	mg/L	800
TS	mg/L	1340 ± 91
TSS	mg/L	63 ± 18
Phosphorous (P)	mg/L	8.9
Solfate (SO_4)	mg/L	55.5
Nitrate (NO_3)	mg/L	< 5
Nitrogen Ammonia (NH_3-N)	mg/L	0.25
Total carbohydrates	mg_{COD}/L	386 ± 7
Soluble carbohydrates	mg_{COD}/L	240 ± 6
Acetone	mg/L	52.85 ± 1.8
Methanol	mg/L	1537.4 ± 48.6
Ethanol	mg/L	18.3 ± 4.8
Propanol	mg/L	2.1 ± 1.9
Butanol	mg/L	0
Acetate	mg/L	182.4 ± 34.4
Propionate	mg/L	0
Butyrate	mg/L	0

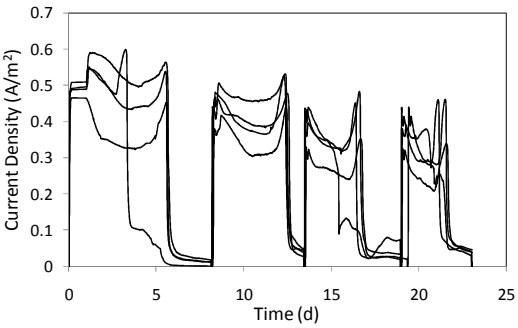

Fig. S.1 - Current density for four representative MFC reactors fed with undiluted, not-amended wastewater.

Tab. S.1 - Chemical characterization of the liquid phase of MFC reactors after the first peak of current production and at the end of the batch cycle.

Parameters			Influent (wastewater)	End of the first current peak (≈2.5 d)	End of the batch cycle (effluent)
pH		-	6.68 ± 0.30	-	7.25 ± 0.22
Conductivity		mS/cm	2.04 ± 0.02	-	2.13 ± 0.13
TCOD		mg/L	4070 ± 180	1460 ± 32	397 ± 11
SCOD		mg/L	3810 ± 160	-	336 ± 20
SCOD/TCOD		%	94 ± 4	-	84 ± 8
TCOD removal		%	-	64	90
Organic Compounds	Acetone	mg/L	52.85 ± 1.8	0	0
	Methanol	mg/L	1537.4 ± 48.6	323.4 ± 24.8	0
	Ethanol	mg/L	18.3 ± 4.8	0	0
	Propanol	mg/L	2.1 ± 1.9	0	0
	Butanol	mg/L	0	0	0
	Acetate	mg/L	182.4 ± 34.4	46.3 ± 14.4	0
	Propionate	mg/L	0	0	0
	Butyrate	mg/L	0	0	0

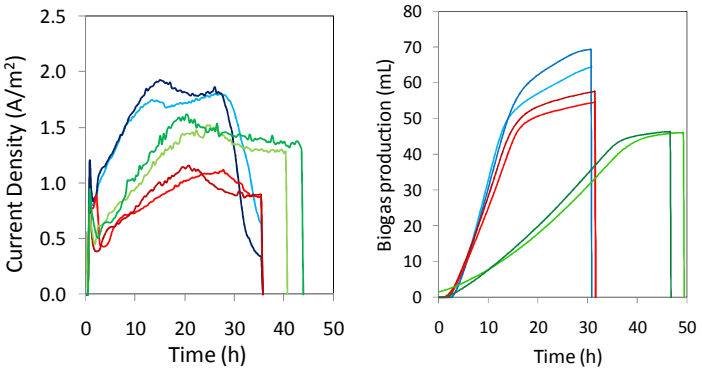

Fig. S.2 - Illustrative cycle of current density (A) and cumulative biogas production (B) in the single chamber MECs used, at 0.7 V applied voltage. Duplicate reactors for each cathode type were used: 10% platinum on carbon cloth (blu lines); 33% MoS_2 on carbon cloth (red lines); bare stainless steel (green lines). (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article).

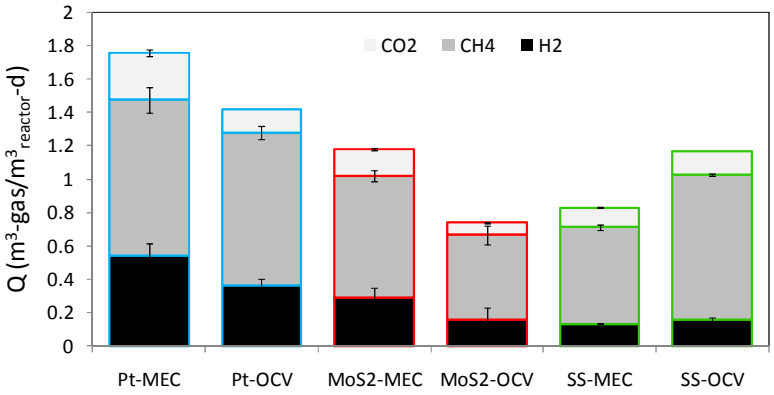

Fig. 1 - Production rate (Q) for specific biogas compound. Comparison between MEC and OCV with different cathodes (data averaged over 5 and 2 cycles, respectively).

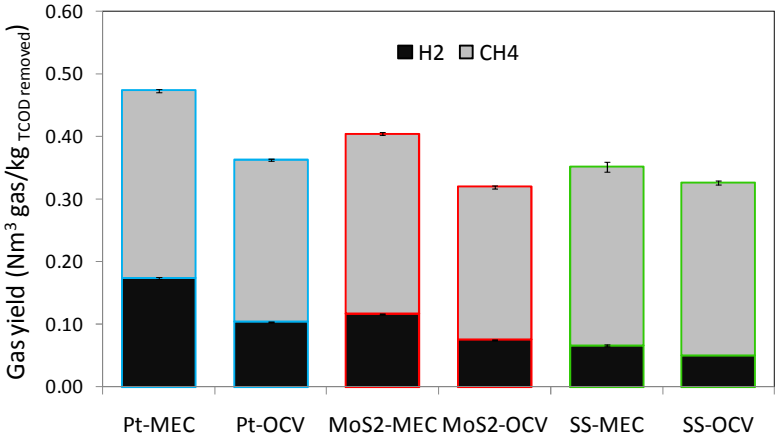

Fig. 2 - Hydrogen and methane yield per TCOD removal. Comparison between MEC and OCV with different cathodes (data averaged over 5 and 2 cycles, respectively).

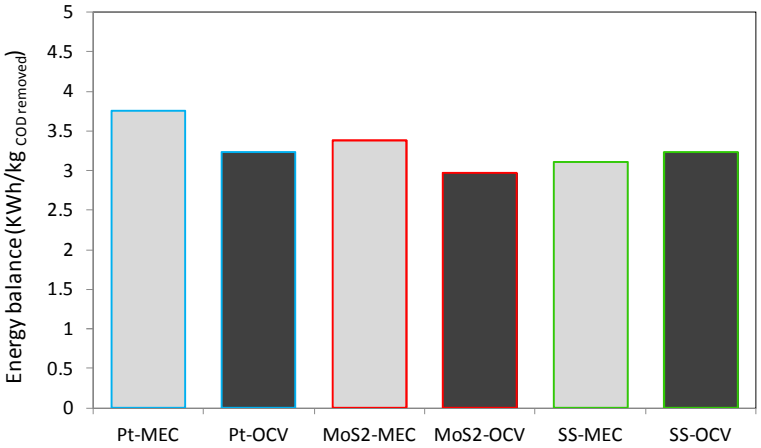

Fig. 3 - Net energy recovery comparison between MEC (grey) and OCV (black) process with different cathodes.

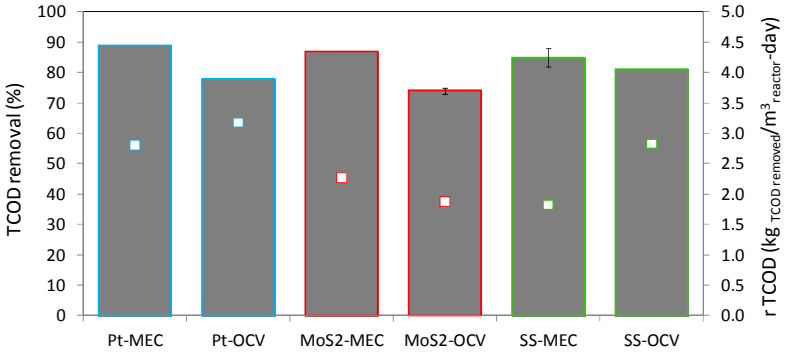

Fig. 4 - TCOD removal (%) and TCOD removal rate ($_{kgTCOD\ removed}/m^3_{reactor}$-day). Comparison between MEC and OCV with different cathodes (data averaged over 5 and 2 cycles, respectively).

Tab. 2 - Effluent characterization and TCOD removal efficiency comparison between MEC and OCV with different cathodes (data averaged over 5 and 2 cycles, respectively).

Parameters			Pt-MEC	Pt-OCV	MoS_2-MEC	MoS_2-OCV	SS-MEC	SS-OCV
pH			6.38 ± 0.02	6.91 ± 0.10	6.32 ± 0.03	6.68 ± 0.21	6.57 ± 0.10	6.34 ± 0.31
Conductivity		mS/cm	2.01 ± 0.04	2.71 ± 0.16	2.05 ± 0.0	2.67 ± 0.04	2.14 ± 0.07	2.16 ± 0.0
TCOD		mg/L	453 ± 3	881 ± 18	513 ± 16	1035 ± 52	585 ± 88	754 ± 31
SCOD		mg/L	356 ± 3	697 ± 35	422 ± 40	832 ± 34	480 ± 46	656 ± 8
TCOD removal		%	89 ± 0	78 ± 0	87 ± 0	74 ± 1	85 ± 3	81 ± 0
Organic Compounds	Acetone	mg/L	0	0	0	8.4 ± 1.3	0	0
	Methanol	mg/L	0	8.8 ± 3.1	0	12.4 ± 5.4	8.6 ± 0.8	0
	Ethanol	mg/L	0	0	0	0	0	0
	Propanol	mg/L	0	0	0	0	0	0
	Butanol	mg/L	0	0	0	0	0	0
	Acetate	mg/L	13.3 ± 1.8	284.0 ± 21.2	27.5 ± 1.5	585.2 ± 25.9	11.5 ± 2.7	131.2 ± 58.9
	Propionate	mg/L	0	20.0 ± 4.4	0	55.6 ± 7.2	0	7.2 ± 1.5
	Butyrate	mg/L	0	0	0	21.2 ± 3.9	0	7.4 ± 2.3

APPENDIX B

Congress communications

Bio-hydrogen production from bio-waste: ready for full-scale applications?

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Abstract

Bio-hydrogen production through fermentation of organic substrates can be a good candidate for full scale application in the next future, especially if the organic materials are by-products or residues of any human activity. In this work, bio-hydrogen was produced from two semi-continuous lab-scale digesters (D1 and D2). The substrates used were household source-separated bio-waste collected in 3 municipalities in Lombardy (Italy) (D1) and the biowaste coming from the green-market of the city of Milan (D2). The two semi-continuous digesters were manually-fed once a day with a organic loading rate of respectively 16.4 and 13.5 g_{VS} d^{-1} l^{-1} and the hydraulic retention times were respectively 4 and 3 days.
Both digesters resulted in interesting bio-hydrogen productions. The average bio-hydrogen production rates were respectively 0.85 and 1.70 Ndm^3 H_2 l digest d^{-1}, while the maximum rates obtained were 1.59 and 3.15 Ndm^3 H_2 ldigest d^{-1}. The average conversion yields were respectively 55 and 126 Ndm^3H_2 $g^{-1}{}_{VS}$, while the maximum rates obtained were 105 and 227 Ndm^3H_2 $g^{-1}{}_{VS}$. The produced biogas showed always complete absence of methane and an hydrogen content around 50%. Because the digesters were manually and semi-continuously loaded, the parameters were not optimized and the producion showed some instability. On the other hand, the obtained

results were indicative and very promising for exploiting this technology in full scale biogas plants.

Keywords: bio-hydrogen, renewable fuel, waste, biogas

1 Introduction

Hydrogen has been always recognized as an ideal alternative energy source to substitute fossil fuels.
Hydrogen produced directly from organic materials by bacteria, i.e. bio-hydrogen, has considerable potential in defining hydrogen's future use [1].
In anaerobic conditions, organic matter is converted to methane and carbon dioxide via a series of interrelated microbial metabolisms, including hydrolysis/fermentation, acetogenesis, and methanogenesis. Fermentative bacteria hydrolyze and ferment carbohydrates, proteins, and lipids to volatile fatty acids, which are further converted to acetate, and CO_2/H_2 by acetogenic bacteria. The products of acetogenesis, i.e. acetate and CO_2/H_2, are finally converted to methane by methanogenic bacteria [2]. A bioreactor could possess significant capacity for the transformation of organics into hydrogen gas when bioactivity of hydrogen consumers contained in a bioreactor was inhibited [3-6]. Some methods have been reported to inhibit methanogen bacteria and to harvest anaerobic spore-forming bacteria such as Clostridium sp., capable to produce hydrogen. One is a heat shock of the inoculum at 100º C for 2 hours, which favours only spore-forming microrganisms. Other method is the pH control in the interval $5<pH<6$, which has been shown to be optimal for hydrogen-type fermentation and to inhibit methanogenic activity [7-9]. In literature, the pH control has been always achieved by the use of chemicals such as NaOH or KOH and HCl [7-10]. On the other hand, the use of large amounts of reagents wouldn't be possible in a full scale-process.

Besides, high concentrations in the digester of volatile fatty acids (VFA), forming during fermentation, are responsible of both inhibiting the hydrogen-producing bacteria and dropping the pH below pH 5 [11]. The concentrations of VFA in the digester are proportional to the organic loading rate (OLR) and at the same time to the hydraulic retention time (HRT). The higher the OLR is, the faster the wash-out of the VFA produced should be, i.e. low HRT.
Han et al. [11] suggested diluting the liquid phase of food waste during its anaerobic fermentation by leaching pure water through the solids (leaching–bed reactor), so that the high concentration of VFA is washed out. The result was an optimal dilution D (D = flow rate / operating volume) of 4.5 d^{-1} [11]. On the other hand, this solution, applied on full-scale, would imply huge water consumption and problems in environmental and economical feasibility.
Many traditional biogas plants in Europe are connected to farms, which produce large amounts of animal slurries. These pre-digested liquid manures, normally have a pH of 6.5-8, a considerable alkaline buffer-capacity and low concentrations of volatile fatty acids.
This paper aims to investigate the use of swine manure in co-digestion with fresh biowaste materials, to control the hydrogen production process in semi-continuous thermophilic bio-digesters. Positive results would help in providing more informations for future full-scale developments.

2 Methods

2.1. Seed microorganisms

The seed sludge was the digestate taken from an anaerobic digester biogas plant and boiled (100º C) for 2 hours to inactivate hydrogenotrophic bacteria and to harvest anaerobic spore-forming bacteria such as Clostridium sp. [7]. This procedure was carried out for the start-up phase of reactors. The pH, alkalinity, total VFA

concentration and volatile solids (VS) concentration of the sludge were respectively 8.1, 7470 mg$CaCO_3$ l^{-1}, 1190 mg l-1 and 750 mg l^{-1}.

2.2. Feedstock for feeding

The two considered substrates were respectively household source-separated bio-waste collected in 3 municipalities in Lombardy (Italy) (S1) and the biowaste coming from the green-market of the city of Milan (S2). The substrates (S) were used for creating feeding mixtures with swine manure (SM). The two mixtures (M1 and M2) were made by mixing at the ratios S:SM of 1:1 and 1:2, respectively. In Table 1, a characterization of the used materials is reported.

Table 1 – Characterization of the materials used

Substrate (S)	S_1	S_2	SM
Type of material	Source separated OFMSW	Fruits-Vegetables (market bio-waste)	Swine Manure
TS content (%)	15.5	10.2	3.2
VS content (%TS)	93.5	89.9	75.3
pH	5.40	3.32	7.65

2.3. Experimental setup and procedure

A completely mixed reactor with working volume of 600 ml was operated in a semi-continuous mode by feeding once a day. The digesters D1 and D2 were operated at a temperature of 55ºC (thermophilic conditions) and Hydraulic Retention Times (HRTs) of 4 and 3 days, respectively (Table 2). The output digestate was

withdrawn once a day before each feed. The digesters were fed with organic loading rates (OLRs) of respectively 16.4 g_{VS} l^{-1} d^{-1} and 13.5 g_{VS} l^{-1} d^{-1} (Table 2).

2.4. Analytical procedures

The biogas production was measured using a volumetric gas meter column connected to the headspace of the digester. Biogas composition was analyzed by a gas chromatograph (Agilent, Micro GC 3000A) equipped with two thermal conductivity detectors (TCD) and two columns, using Nitrogen and Helium as carriers.

3 Results and discussion

3.1. H_2 productions

The process performances in terms of H_2 yield per S unit added to the digester (Ndm^3 H_2 $kg^{-1}{}_{VS}$) and the volumetric hydrogen production rates (Ndm^3 H_2 $kg^{-1}{}_{VS}$) are reported in Table 2. The H_2 to CO_2 ratios in biogas were always found in the range 40 – 60% in both the digesters. Methane was never found in the biogas, indicating complete inhibition of the hydrogen-consumers microrganisms. These favourable conditions were created by the quick HRTs (3-4 days) and the relatively low pH operational conditions (Table 2).
The hydrogen production rates and yields showed noticeable imbalances. Some intermediate periods, for both the digesters, were characterized by a lag-phases, in which less biogas was produced compared to the maximum productions. This drove to low hydrogen yields and rates, if compared with the best results (Table 2). This was the reason for the relatively big differences between the average and the maximum values for both H_2 production yields and rates reported in Table 2. However, the semi-continuous mode, do not permit to achieve stable productions and to optimize the results. On the other hand, the results were quite satisfactory, if compared to the yields of

43 dm^3H_2 $kg^{-1}_{VSadded}$, achieved by continuously stirred and fed reactors (HRT of 2 days) by Liu et al. [7].

4 Conclusions

Bio-hydrogen production from different types of bio-waste was found possible. With simple lab-equipment, satisfactory H_2 productions were achieved. The use of swine manure in co-digestion with the bio-waste favoured the process conditions, by buffering the pH to ideal values and diluting the produced VFAs. Further research should be carried out in improving and optimizing the process and for assessing the methane yields, obtainable from the effluent of the H_2-production. This may easily lead to full-scale applications in a close future.

Table 2 – Operational conditions and bio-hydrogen productions

	D_1	D_2
Type of substrate (S)	Source separated OFMSW	Fruits-Vegetables (market bio-waste)
Mix with swine manure (S:SM)	1:1	1:2
HRT (d)	4	3
OLR (g_{VS} l^{-1} d^{-1})	16.4	13.5
Operational pH	5.75	4.85
H_2:CO_2 ratio in the biogas (%)	40 – 60 %	40 – 60 %
H_2 Yield (Ndm^3 $kg_{VS\ added}$)	Average: 55	126
	Maximum: 105	227
H_2 Rate (Ndm^3 l^{-1} d^{-1})	Average: 0.85	1.70
	Maximum: 1.59	3.15

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An operational strategy to produce Bio-hydrogen: the use of digestate for process control

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Abstract

A semi-continuous digester was fed twice a day with a concentrated solution of glucose (100 g l^{-1}) and monitored for a 30-days period, with the aim of testing the possibility of utilizing the digestate of a traditional biogas plant, after a heat-shock at 100ºC, for controlling process parameters (organic loading rate OLR, pH, volatile fatty acids VFA concentration), by adding it to the fresh substrate at a ratio R of the total feeding volume. The process resulted instable for OLR=10 g_{VS} $L^{-1}$and R=0.7, while more stable for OLR of 5 g_{VS} $L^{-1}$and R=0.85. The maximum bio-hydrogen production rate in stable conditions was 100 $NmLH_2$ h^{-1} and the conversion yields were 1.7 - 1.8 $molH_2$ mol^{-1}glucose. The produced biogas showed always complete absence of methane.

Keywords: bio-hydrogen, renewable fuel, waste, biogas

1 Introduction

Hydrogen has been always recognized as an ideal alternative energy source to substitute fossil fuels.
Hydrogen produced directly from organic materials by bacteria, i.e. bio-hydrogen, has considerable potential in defining hydrogen's future use [1].
In anaerobic conditions, organic matter is converted to methane and carbon dioxide via a series of interrelated microbial metabolisms,

including hydrolysis/fermentation, acetogenesis, and methanogenesis. Fermentative bacteria hydrolyze and ferment carbohydrates, proteins, and lipids to volatile fatty acids, which are further converted to acetate, and CO_2/H_2 by acetogenic bacteria. The products of acetogenesis, i.e. acetate and CO_2/H_2, are finally converted to methane by methanogenic bacteria [2]. A bioreactor could possess significant capacity for the transformation of organics into hydrogen gas when bioactivity of hydrogen consumers contained in a bioreactor was inhibited [3–6]. Some methods have been reported to inhibit methanogens and to harvest anaerobic spore-forming bacteria such as Clostridium sp., capable to produce hydrogen. One is a heat shock of the inoculum at 100ºC for 2 hours, which favours only spore-forming microrganisms. Other method is the pH control in the interval $5<pH<6$, which has been shown to be optimal for hydrogen-type fermentation and to inhibit methanogenic activity [7 - 9]. In literature, the pH control has been always achieved by the use of chemicals such as NaOH or KOH and HCl [7-10]. On the other hand, the use of large amounts of reagents wouldn't be possible in a full scale-process.

Besides, high concentrations in the digester of volatile fatty acids (VFA), forming during fermentation, are responsible of both inhibiting the hydrogen-producing bacteria and dropping the pH below pH 5 [11]. The concentrations of VFA in the digester are proportional to the organic loading rate (OLR) and at the same time to the hydraulic retention time (HRT). The higher the OLR is, the faster the wash-out of the VFA produced should be, i.e. low HRT.

Han et al. [11] suggested diluting the liquid phase of food waste during its anaerobic fermentation by leaching pure water through the solids (leaching–bed reactor), so that the high concentration of VFA is washed out. The result was an optimal dilution D (D = flow rate / operating volume) of 4.5 d^{-1} [11]. On the other hand, this solution, applied on full-scale, would imply huge water consumption and problems in environmental and economical feasibility.

Traditional biogas plants produce abundant effluents, i.e. digestates, which have normally a pH of 7-8, a considerable alkaline buffer-capacity and low concentrations of volatile fatty acids, as they were transformed into methane.
This paper aims to investigate the use of pre-heated (100ºC) digestates to control the hydrogen production process in a semi-continuous thermophilic bio-digester, by diluting the liquid medium and buffering the pH to the desired values.

2 Methods

2.1.Seed microorganisms

The seed sludge was the digestate taken from an anaerobic digester biogas plant and boiled (100ºC) for 2 hours to inactivate hydrogenotrophic bacteria and to harvest anaerobic spore-forming bacteria such as Clostridium sp. [7]. This procedure was carried out twice a day, before feeding. The pH, alkalinity, total VFA concentration and volatile solids (VS) concentration of the sludge were respectively 8.1, 7470 mg $CaCO_3$ l^{-1}, 1190 mg l-1 and 750 mg l^{-1}.

2.2.Feedstock for feeding

A solution of 100 g l^{-1} of pure glucose (99%) was used as feeding mixture, in order to represent an extreme condition for concentration of sugar-type substrate in the feedstock. The glucose was also chosen as known substrate for better understanding the process performances.

2.3. Experimental setup and procedure

A completely mixed reactor with working volume of 600 ml was operated in a semi-continuous mode by feeding twice a day a mix of the glucose solution and the heat-shocked digestate by a syringe. The

digester was operated at a temperature of 55ºC (thermophilic conditions) and a HRT of 3 days. Output digestate was withdrawn twice a day before each alimentation.

2.4. Experimental conditions

Two experiments were performed to test the process behaviour in producing biohydrogen. The aim was to find the maximum OLR and the minimum recirculation ratio (R), i.e. the digestate input volume divided by the total input volume. The digester was fed in the first two-week period with an OLR of 10 gVS l^{-1} d^{-1} and in the second period with an OLR of 5 gVS l^{-1} d^{-1}. The recirculation ratios were of 0.7 and 0.85 respectively.

2.5. Analytical procedures

The biogas production was measured using a volumetric gas meter column connected to the headspace of the digester. Biogas composition was analyzed by a gas chromatograph (Agilent, Micro GC 3000A) equipped with two thermal conductivity detectors (TCD) and two columns, using Nitrogen and Helium as carriers.

3 Results and discussion

3.1. H_2 production

In Figure 2 are reported the results of the process performance in terms of H_2 production per mole of glucose added and the hydrogen production rate. Methane was never found in the biogas, indicating complete inhibition of the hydrogen-consumers microrganisms. Figure 3 shows the pH trend and the total VFA concentration in the digester.

3.2. First two-week period (OLR=10 gVS l^{-1} d^{-1})

The first period (hours 0 to 250) was characterized by a lag-phase during the first week (hours 0 to 115), in which few biogas was produced (Figure 1). This drove to low hydrogen yields, if compared with the second week, when the process yielded 1.5-1.8 $molH_2$ mol^{-1}glucose. This result was quite satisfactory, if compared to the maximum yields of 2.45 and 2.6 $molH_2$ mol^{-1}glucose achieved in controlled batch cultures by Van Ginkel et al. and Taguchi et al. [12 - 13]. Hang-sik and Jong-Ho [10] obtained a calculated value of 2.2 $molH_2$ $mol^{-1exose}$, feeding mixed food waste continuously, with an operative HRT of 5 days and adjusting the pH with specific pure reagents (KOH and HCl).

The hydrogen production rate showed notable imbalance, showing, during the second week, maximum peaks around 150 and 200 mlH_2 l^{-1} $h^{-1}$and null values. The obtained maximum rates were four times higher than those measured by Hang-sik and Jong-Ho [10], in fermenting mixed food waste, with similar OLR. This was probably caused by the high availability of glucose to microrganisms, if compared to more complex organic molecules that must be hydrolyzed before fermentation.

On the other hand, the use of glucose meant higher shock due to the high-loading. The fast production of VFA partially inhibited the fermentation because of high concentrations (around 10 g l-1) (Figure 1) and dropped twice the pH to 4.7 and 4.8 (Figure 2), causing process imbalance. The addition of digestate in the feeding with the ratio R=0.7, resulted non-sufficient to control the process parameters. Because the digester was semi-continuously fed, this effect was probably more evident than what would happen in a continuously-fed system.

3.3.Second two-week period (OLR=5 gVS l-1 d-1)

From hour 250 to 500, the OLR was lowered to 5 g_{VS} l^{-1} d^{-1}. The hydrogen production gave yields almost constantly around 1.7 – 1.8 $molH_2$ mol^{-1}glucose and rates following a relatively stable trend, with maximum peaks around 80 mlH_2 l-1 h^{-1} (2). The variations were probably caused by the semi-continuous feeding. The pH was maintained always higher than in the first period (Figure 2), between pH 5.4 and 6.2, meaning that the R ratio (0.85) was the upper limit for a satisfactory control of the process pH. The VFA concentration resulted in lower values than in the first period (Figure 2).
These results revealed that the digestate added to the feeding (at the ratio R) has a remarkable effect on the process control, for both diluting VFA concentration and buffering the pH to desired values (5<pH<6). This strategy would probably work better in a continuously-fed system.

4 Conclusions

The studied semi-continuous process, with a fixed HRT of 3 days, showed imbalance conditions for R between 0.7 and 0.85 and for OLR between 5 and 10 gVS l^{-1} d^{-1}. Comparing this process to the one proposed by Han et al. [11], the dilution ratio (D) for optimal operation, in the present case, would be 0.3 d^{-1}. Using pure water in a leaching-bed system, Han et al. found optimal Ds between 2 and 5 so that high water consumptions were needed. The use of digestate instead of water would probably give a better option to both control the pH and dilute the VFAs, also in a leaching-bed system. Further research should be carried out to test this strategy for fermenting various kinds of organic substrates, with different operational conditions.

Fig. 1. Process yield and hydrogen production rate.

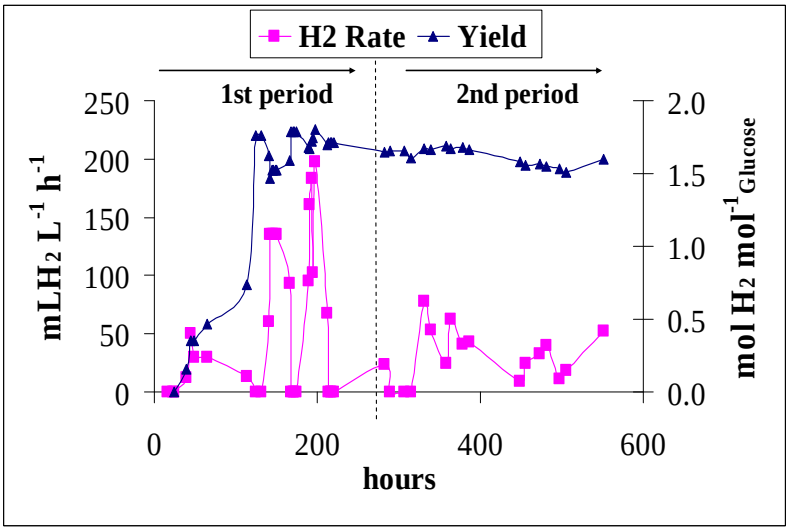
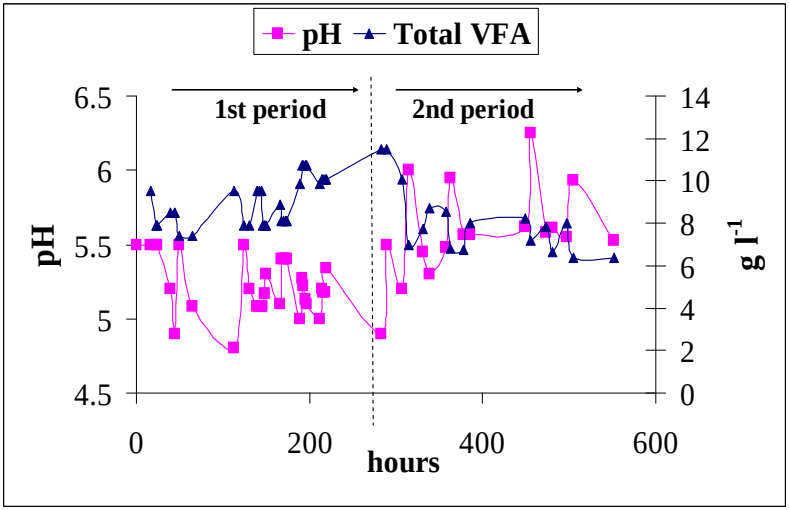


Fig. 2. Trends of the pH and the total VFA concentrations, measured during the test.



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