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Biohydrogen production by dark fermentation: from laboratory to full scale

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1. INTRODUCTION

The need for energy worldwide has been increasing exponentially, especially in these last years; the reserves of fossil fuels have decreased, and their combustion has serious adverse effects on the environment due to CO_2 emissions.

Fossil fuels are causing massive climate changes and are thus seriously upsetting the ecosystem in all world regions, in particular considering the modification in the average temperature of Earth's atmosphere, a phenomenon influenced by many factors, included greenhouse effect.

To some extent, this is a natural phenomenon meant to heat the planet's surface, but over the last decades human activities have become the major contributor to a worrying increase of this process.

After the Industrial Age, human activities caused the atmosphere's composition to change. While the most abondant atmospheric gases (nitrogen and oxygen) are not involved in the increase of greenhouse effect, other compounds such as CO_2, methane, nitrogen oxides absorb infrared radiations and contribute to the effect.

To reduce greenhouse gases emissions a transition to large-scale production of energy from renewable sources is required.

This step is not yet feasible in the short term, because the current state of technology to produce renewable energy is not competitive compared to fossil fuels.

Before obtaining a significative transition to these technologies is therefore necessary to ensure affordability and thus lower production costs.

For these reasons, many researchers have been working to explore new sustainable energy sources to replace fossil fuels.

In ordert to cope with climate change, in 2007 the European Union adopted an energetic plan, knowk as "20-20 by 2020 plan", wich states:

- an independent EU commitment to achieve a reduction of at least 20% of greenhouse gas

emissions compared to 1990 levels by 2020, and the goal of reducing emissions by 30% by 2020, subject to the conclusion of an international agreement on climate change;

- a binding target for the EU of 20% of energy from renewable sources by 2020, including a 10% target for biofuels.

Renewable energy sources can be used in a continuous way withous exhaustion and can renew their availability in short time; unlike fossil fuels, their use produces less environmental pollution.

According to italian regulations ((DL 29 dicembre 2003, n.387, Art.2), the following can be considered renewable sources:

- Solar energy (thermal and photovoltaic);

-Hydropower;

-Wind energy;

-Wave energy;

-Tidal energy;

-Geothermal energy;

-Energy from biomass (biogas, vegetal oils and biodiesel, bioethanol, chips).

Biohydrogen production from microbial anaerobic digestion allows to obtain a high-quality fuel with very low dangerous emissions (its combustion only produces water).

1.1 Energy from biomass

The concern about the instability of supply of fossil fuels, the limits of their reserves and, not least, environmental pollution and climate change have brought a new vision of the use of biomass for biorefinery concepts where biomass is used as a raw material in place of fossil fuels for the production of biofuels, chemicals, solvents, etc. by biological conversion processes.

Biomass is the natural, more complex form of solar energy storage.

This, in fact, allows the plants to convert atmospheric carbon dioxide into organic matter through the process of photosynthesis.

Biomass also has the important property of preserving intact its energy until it is used, although in general it has a moderate calorific value. The use of biomass as an energy source is considered "clean" because it is assumed that the inorganic carbon produced by combustion is then fixed into organic carbon through photosynthesis during the reforming of biomass that need to be restored in order to achieve a carbon balance and zero net emissions.

The term “biomass” refers both to energy crops and byproducts as wastes, manure, vegetable and pruning wastes, organic fraction of municipal wastes and many more, suitable for a forther energy esctraction.

The main energy uses of biomass are aimed at the direct production of energy, usually by combustion (bioenergy), the synthesis of biofuels and the synthesis of solid products derived from the fibers present in the biomass (building materials, bioplastics ...).

Biomass can be exploited through processes of biochemical conversion (for biomass with C/N ratio of less than 30 and humidity higher than 30% when collected) which allow to obtain energy from chemical reactions with the help of enzymes, fungi and micro-organisms. If C/N ratio is higher than 30 and humidity is low, as in ligno-cellulose rich products, thermochemical conversion processes are preferred.

Regarding biofuels, ethanol, which can be used as fuel for internal combustion engines in lieu of gasoline, can be derived by fermentation of plants rich in sugar, such as sugar cane, beet and corn. By squeezing of oil-rich plants (sunflower, soy, rapeseed) biodiesel is obtained.

Some types of biomass, such as wood, do not need to undergo treatment; others, such as vegetable or municipal waste, must be processed, for example in a digester.

Biomass also have limitations that are related to their own production:

-Availability: with the exception of solid municipal wastes, cultivated biomass (crops) are not available throughout the year and therefore require large areas for the storage of material;

-Yield per hectare: in contrast to traditional fuels, which are generally found in large deposits, the production of biomass generally occurs on wide areas and this is perhaps the main limitation to the

use of biomass.

Since conventional energy resources (oil and natural gas) are being depleted, it is necessary to exploit these new alternative energy sources through a policy that encourages research and development.

1.2 Anaerobic digestion (AD)

Methane formation is a biological process that takes place naturally when the organic material (biomass) is decomposed in a humid atmosphere and in absence of oxygen by a group of metabolically active microorganisms (methanogens). Methane gas, poorly soluble in water, passes to the gaseous phase, while the carbon dioxide is distributed in the gas phase and in the liquid.

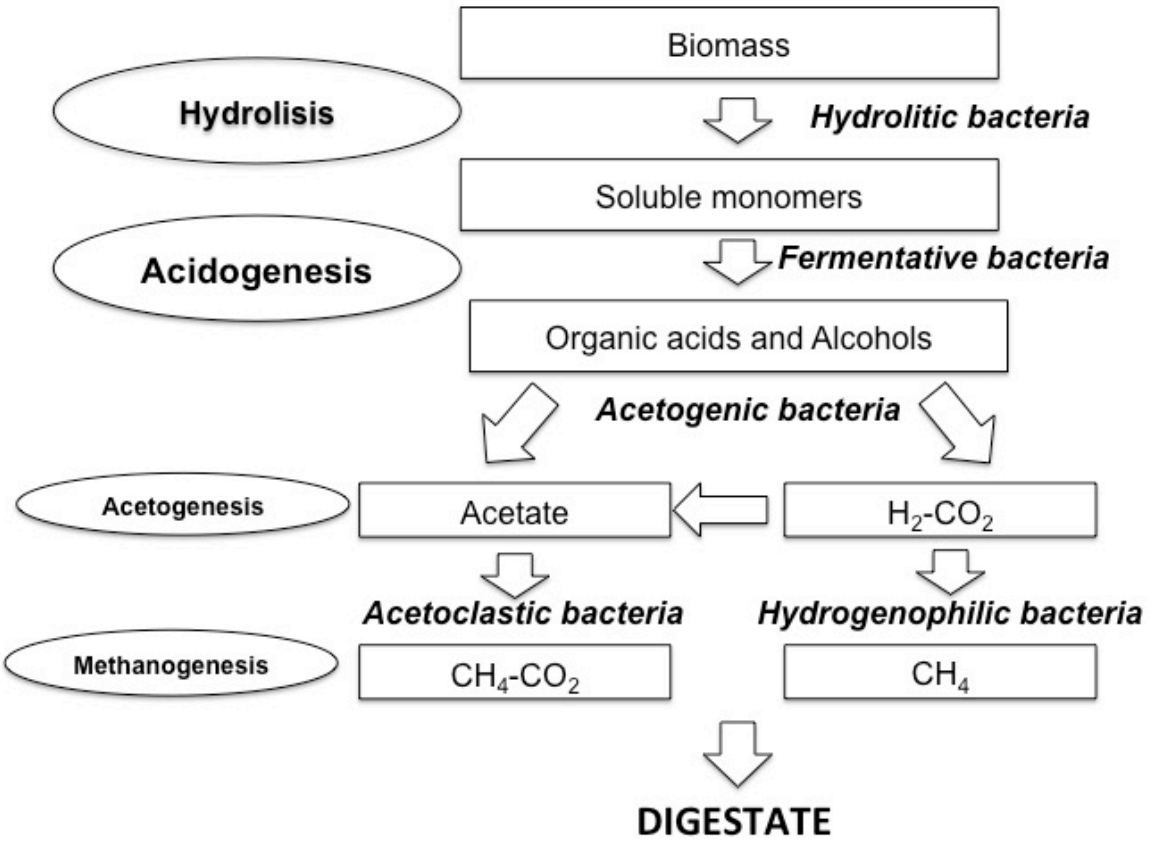

Fig.1.1: AD phases

Degradation and hydrolysis.

Degradation and hydrolysis are processes that lead to the breaking and solubilization of complex organic molecules to soluble substrates. The starting substrates are complex mixtures of particulates, macromolecules of carbohydrates, proteins and lipids. The degradation, therefore, includes a series of steps as the lysis, the non-enzymatic decomposition, phase separation and rupture molecular physics. The process is catalyzed by enzymes that degrade carbohydrates, proteins and lipids, respectively, to monosaccharides, amino acids and long chain fatty acids.
The enzymatic hydrolysis is actually a complex multi-stage process for carbohydrates, proteins and lipids, which may include the production of multiple enzymes, and the steps of diffusion, adsorption, reaction and enzymatic inactivation.

Acidogenesis

The degradation of soluble sugars and amino acids, resulting from the previous hydrolytic step to a series of simpler compounds.
Given that the yields in free energy are usually quite high, acidogenic reactions can occur at high concentrations of hydrogen or formate and at rather high biomass levels.
Acetate, propionate and butyrate are the major end products of monosaccharides' acidogenesis and will be degraded differently from subsequent reactions.
Lactate and ethanol are two intermediates of the digestion process, in particular with regard to the great influence of pH on the production of hydrogen (Zheng e Yu, 2005; Chen et al., 2002).
In fact lactic acid has a low pKa (3.08) and a large effect on pH; ethanol, on the other hand, with its lower pKa value and less influence the pH, is significantly present in the production of biohydrogen as a product of direct monosaccharides degradation and often as an alternative acetate at low operative pH values (pH < 5; Ren et al., 1997).
The lactate is then further degraded, always for acidogenesis, to propionate and acetate.

Acetogenesis

Starting from the substrates formed during the hydrolysis and acidification steps, acetogenic bacteria produce acetic and formic acid, carbon dioxyde and hydrogen.

During the production of acetic acid, the presence of molecular hydrogen in the medium can lead to problems of inhibition.

Methanogenesis

The production of methane can occur through two different pathways of reactions:

- dismutation of acetic acid by anaerobic acetoclastic bacteria with formation of methane and carbon dioxide

-by hydrogenotrophic bacteria, with the reduction of carbon through anaerobic oxidation of hydrogen to methane.

As was previously mentioned, the hydrogen and formate created in the acetogenic phase must be kept at low concentrations and thus are consumed by the methanogenic microorganisms.

With their activities, methanogenic bacteria have two important functions in the anaerobic food chain: they degrade acetic acid and formic acid to methane by removing acids from the medium and thus preventing the inhibition of degradation of the substrates organic due to excessive acidity, and on the other hand they maintain the hydrogen concentration at low levels so as to allow the conversion of long chain fatty acids and alcohols to acetate and hydrogen. In fact, if hydrogenotrophic pathway is slowed down, an accumulation of hydrogen in the mean is observed, with consequent inhibition of methane production, while the way acetoclastic pathway can undergo phenomena of substrate inhibition in presence of high concentrations of acetic acid.

Inside a reactor, therefore, the low energy yield of methanogenesis forces the involved microorganisms to co-operate very efficiently and to establish sintrophic relationships, a thermodynamical sinergy. This is defined as the cooperation between two organisms in which both depend on each other and this mutual dependence can not be replaced by the addition of nutrients.

Anaerobic digestion can be used for production purposes through bio-reactors, structures to nurture and maintain a productive and viable bacterial consortium in able to degrade continuous biomass to obtain biogas.

The production of methane by anaerobic fermentation of sewage and waste (including pig slurry, manure, and the organic fraction of separated waste) is a process already widely applied. In this system, hydrogen is an intermediate of the process which, however, is not available as it is quickly used and converted to methane by methanogenic microorganisms.

1.3 Hydrogen

The interest around the hydrogen was born in the early 70s after the first oil crisis and growing concerns about the environment, seeing significant benefits in terms of improved air quality and reduction of energy dependence on oil imports.

That interest quickly subsided after the decline of oil prices in the mid-80s, and then reappear in the early 2000s, driven in particular by the search for energy strategies to reduce greenhouse gas emissions and the new surge in energy prices fossil.

1.3.1 Characteristics

Hydrogen has the following characteristics:

- It is the most present element in the universe, constituting three-quarters of all matter, but in a free form it represents only 0.07% of atmosphere and 0.14% of the earth's surface;
- Has a high calorific value (3042 cal/m3) and the highest energy content per unit mass of all the known fuel (143 GJ / t);
- Is the only common fuel is not chemically bound to carbon and can be used for energy generation in technologies characterized by a very low rate of emissions. Especially in fuel cells, a particular form of electrolytic cell, hydrogen can generate heat and electricity with only emission of water vapor, which can be recycled to produce additional hydrogen(Nath e Das, 2003). Its combustion is

thus free of emissions of oxides of carbon, even if the high temperatures produce high rates of nitrogen oxides (Zurawski et al., 2005). In conclusion, it is generally harmless to human health and environment, since it does not contribute to the greenhouse effect, the consumption of ozone and acid rains.

1.3.2 Hydrogen in fuel cells

Currently the principal use of hydrogen is the synthesis of ammonia, which absorbs 49% of the production, 37% is employed in the processes of petroleum refining, 8% for the production of methane and about 6% for the production of various substances (Stiegel and Ramezan, 2005). A promising technology for the use of hydrogen is that of the fuel cells (fuel cells, FC), able to directly transform the energy of the fuel into electrical energy by electrochemical pathway. Even in devices of small size (on the order of 10 kW), this process achieves higher yields than the thermodynamic cycles used in conventional conversion systems, comparable to the best generation technologies currently used in large power plants (combined cycle). The high electrical yields lead to savings in terms of primary resources (fuel), but also to a reduction in emissions of greenhouse gases (carbon dioxide) and a substantial elimination of pollutant emissions. A fuel cell is an electrochemical generator in which a fuel (typically hydrogen) and an oxidant (oxygen or air) enter and it produces continuous electric current, water and heat. Differently from common batteries, in the fuel cell, the active material is renewed continuously and therefore the direct electric current can be delivered indefinitely if you keep the supply of fuel and oxidant gases.

An aspect of fundamental importance for the applications of fuel cells, is represented by the fact that the effluents (water and exhaust gases), which must be continually removed from the cell, do not contain pollutants and are not harmful to the environment. Despite its enormous potential, fuel cells techinolgy is not yet considered mature: performances are to be improved and production costs are not yet compatible with commercial applications of reference.
Even if it is currently being used as fuel for rocket motors, there is no doubt that in the future the

greater use of hydrogen will reside in the transport sector. In fact, in addition to reducing the emission of pollutants, hydrogen fuel cells show yields two to three times greater than the current gasoline-powered engines (Nath e Das, 2003).

1.3.3 Hydrogen production

Although hydrogen is the most abundant element in the universe, it does not exist naturally in large quantities or concentrations on Earth, but it must be produced from other substances, such as fossil fuels, water, biomass, etc. Currently, hydrogen can be produced in different ways.

From fossil fuels:

- Steam reforming;
- Thermal cracking;
- Partial oxydation;
- Coal gasification;

From biomass:

- Pyrolisis;
- Gasification;
- Microbial conversion;

From water:

- Electrolysis;
- Photolysis;
- Thermochemical processes;
- Thermolysis or direct thermical decomposition;

The biological production of hydrogen seems to be particularly promising: it is a set of those technologies that use microorganism-lead processes.

This kind of process shows several advantages:

- It primarily operates at temperature and pressure valuse similar to ambiental ones (30-50 ° C, 1

atm) and has therefore a low energetic impact;

- It Is a process of considerable environmental compatibility pointing to tread a new path for the use of inexhaustible energy resources;

- Can take advantage of various waste materials, facilitating the reuse of waste, at least in their organic fraction.

This technology fits perfectly among the strategies of sustainable development, since it is an integrated technology that combines the energy recovery with waste treatment (Li, 1999).

Considering that the reserves of fossil fuels (especially oil) are being consumed at an alarming rate, the production of hydrogen through the exploitation of alternative sources seems to be an imperative for the immediate future.

1.3.4 Hydrogen from biomass

Biomass is the most versatile renewable source and can be used, as seen above, for the production of biohydrogen(Nath e Das, 2003). The biomass has the fundamental characteristic of being a renewable source, but thanks to its versatility the list of species of plants, of the intermediates and of waste materials potentially suitable as a substrate is almost unlimited.

The main biomass resources include agricultural crops and their waste products, ligno-cellulosic products such as wood and wood waste, waste from food processing, algae and aquatic plants and waste products in anthropic environments.

Numerous processes allow the production of hydrogen from biomass:

1. Thermochemical gasification coupled to the reaction of "water-gas shift";
2. Fast pyrolysis followed by reforming of bio-oil carbohydrates fractions ;
3. Solar direct gasification;
4. New and different gasification processes;
5. Conversion of syngas derived from biomass;

6. Supercritical conversion;

7. Microbial conversion .

The latter process is widely varied, depending on the microorganisms and the physical and metabolical conditions in which H_2 production occurs.

Some microorganisms in nature can in fact produce hydrogen: through biotechnology is not only possible to take advantage of the work of these microorganisms, but also change the key enzymes through genetic engineering techniques or transfer such components to more efficient and productive bacteria.

Overall, the microbial production of hydrogen can be classified as follows:

-Direct and indirect biopyrolysis of water through algae and fotobacteria;

- *Microbial water shift reaction*

- Photodegradation of organic compounds by photosynthetic bacteria;

- .Hydrogen production by fermentation of organic compounds;

- Hybrid systems using photosynthetic and fermenting bacteria.

In general, if the organic compounds are the sole source of carbon and energy to provide metabolic energy, the process is called "dark fermentation", but if additional light energy is required, the process belongs to the category of photobiological processes.

1.4 Hydrogen from AD

Biological production of hydrogen by microbial fermentation of biomass at first was not considered promising by scientists, when compared to photosynthetic techniques, despite its general lower complexity(Zurawski et al., 2005).

Das and Vaziroglu (2001) and Nath and Das (2004) point out three factors in favour of the fermentative process:

1. the fermentative bacteria have very high production rates of hydrogen;

2. these bacteria can produce hydrogen from organic substrates steadily, day and night, not

requiring additional light.

3. they have good growth rates without suffering the inhibitory effects of oxygen.

The current growing worldwide interest in the "dark hydrogen fermentation" is even more evident when considering that:

-all power plants in the past were centralized systems with a power of not higher than 30 MW; on the contrary today the development of fuel cells has made decentralized systems more attractive. In this case, power plants can be located close to sources of raw material, reducing the cost for material transport;

- Hydrogen and methane can be produced by a consortium of microorganisms, using different sources of carbohydrate;

- The carbon dioxide resulting from the process is emitted exclusively in the production site, which facilitates its subsequent use;

- The accumulation of knowledge and advances in genetic research may allow a better control of cellular metabolism.

The term “fermentation “generally indicates a process in which the initial organic compound is partly oxidized and partly reduced. In absence of electron acceptors supplied from the outside, balanced redox reactions of organic compounds are carried out, with release of energy. (Brock et al., 1996).

Microbial hydrogen production is a ubiquitous phenomenon in conditions of anoxia or anaerobically, or in the absence of oxygen as the electron acceptor. A large variety of bacteria uses the reduction of protons to hydrogen to eliminate the reducing equivalents derived from the primary metabolism. In aerobic conditions, oxygen is reduced to water; in anaerobic environments, other elements must act as electron acceptors(Nandi and Sengupta, 1998).

Despite the microbial production of hydrogen is a ubiquitous phenomenon, generally the release of hydrogen from organic waste batteries or sewers is not evident. The reason is that in natural environments, numerous bacteria consume hydrogen, using H2 as a source of reducing power; for

this reason in nature is not normally possible to witness a net production of hydrogen.

1.4.1 Hydrogen-producting microorganisms

From a practical point of view, controlling fermentative microorganisms means maximizing the amount of hydrogen producible. Even isolation and possible enrichment steps are delicate processes, as much as the correct choice of the composition of the culture medium anaerobic. Hydrogen-producing microorganisms can be divided into three categories (obligate anaerobes, facultative anaerobic and aerobic) on the basis of their dependence on oxygen. Obligate anaerobes are organisms that do not require oxygen for their vital functions, do not use it as an oxidizing agent for the demolition of nutrients and can not live in the presence of oxygen. Microorganisms of the genus *Clostridium* were found to be dominant in the process of anaerobic fermentation of hydrogen. These organisms are anaerobic bacilli, capable of forming spores in the case of adverse environmental conditions, ubiquitous (present in the soil, water, sewers ...) and for the most part are harmless forms of saprophytes.

The facultative anaerobic microorganisms are resistant to oxygen (and therefore able to live both in the presence and absence of O2), they can quickly consume oxygen, restoring anaerobic conditions inside the fermenter. This feature represents a major technical advantage of facultative anaerobes compared to obligate anaerobes: the latter, being very sensitive to oxygen, often do not survive in minimal concentrations of O2. *Enterobacter* is the most abundant genus among the facultative anaerobes, it has high rates of growth, using a wide range of sources of carbon and its hydrogen production is not inhibited by high partial pressures of H2. Compared to *Clostridia*, however, it normally provides lower yields in H2/mol mol glucose.

With the term “termophilic” refers to a collection of organisms, belonging to the broader class of extremophiles, which can live and multiply at relatively high temperatures, i.e. above 45 ° C. The ideal habitat of thermophilic is represented by the regions of the Earth characterized by geothermal activity, as in the case of thermal waters and estuaries of deep sea hydrothermal vents, and where

there is decaying organic matter, as in the case of peat bogs and compost. Thermophiles can be either forced and optional: obliged termophilic bacteria necessarily require high temperatures in order to grow, while facultative ones can develop both at high and lower values of temperature.
In case of the isolation of microflora from different sources (eg soils, anaerobic digesters or from organic waste and sludge from waste water of the kitchens) the obtained population will be a mixed colture. This system usually requires the preparation of enrichment cultures by forced aeration of sludge or heat treatment to inhibit the activity of hydrogen-consuming microorganisms present and ensure the survival of anaerobic bacteria spores. Considering how likely contamination of pure coltures is, the use of mixed cultures obtained from organic waste seems to be particularly advantageous for purely industrial applications.

1.4.2 Substrate for AD

The two main aspects to consider when choosing a substrate to produce hydrogen through dark fermentation are the range of organic compounds availability and the quality of the material used. From a thermodynamic point of view it is preferred the conversion of carbohydrates to organic acids and hydrogen, as this ensures the highest yields of hydrogen per mol of substrate. These carbohydrates can be monosaccharides (glucose, isomers of hexoses, etc..) but also polymers. Considering the large number of microbial species able to produce hydrogen, it is possible to generalize that most of the carbohydrates are a suitable substrate to dark fermentation while proteins, peptides and amino acids are less adequate.
According to some studies ((Noike and Mizuno, 2000; Yu et al., 2002) different forms of organic wastes are usable, from solid ones like hay to liquids like industrial wastewaters. The use of waste and sewage rich in carbohydrates and low in nitrogen from the agricultural and food industries seems a viable option, considering the problem of the cost of raw materials.
It is expectable that the production of economically interesting substrates will require, in time, the development of methods of pre-treatment with low-cost and low energy demand.

it is clear that from a practical and applicative point of view waste products containing cellulose, easily degradable sugars (such as sawdust or prunings and clippings) and in expansion with the increase of industrial and agricultural processes are preferable to substrates pure glucose and sucrose.

Tsygankov (2007) suggests in particular two distinct strategies for the treatment of waste cellulose for the production of hydrogen: the integration of the processes of degradation of cellulose and production of hydrogen in a single bioreactor or preliminary hydrolysis (chemical or enzymatic) of waste to give sugars, followed by the transfer of output to bioreactor.

1.4.3 Process variables

Much of the latest research on the possibility of maximizing the production of hydrogen by fermentation have focused on the optimization of the process and the determination of the best choices of sources of inoculum, pretreatment methods of inoculum itself, of fermentable substrates and environmental conditions of the reactors.

The main possibilities for intervention to improve the fermentation process is the adjustment of the variables that affect microorganisms.

Nutritional limitations

First of all, cell growth can be restrained by nutritional limitations, leading to higher yields of hydrogen through the increase in catabolic activity. Putting the coltures under unbalanced nutrition conditions can lead to growth difficulties for the microflora, but at the same time it can prolong the conversion of the substrate to hydrogen (Benemann, 1996).

Thermical shock

Zurawski et al. (2005) analyzed the effect of heat shock pre-treatment on the microflora of sludge from waste water, a strategy to inhibit the bioactivity of hydrogen-consuming bacteria, such as methanogens, and to enrich the concentration of spore-producing bacteria. In fact, most of the hydrogen-producing bacteria can form endospores in the presence of unfavorable environmental

conditions (temperature, chemical toxicity, etc.).. The treatment is carried out in a water bath at 80 ° C for 30 minutes.

Other authors such as Kim et al., 2004 tried a thermical shock at 90°C for 10 minutes, while Van Ginkel et al., 2001 tried 104°C for 120 min.

pH

The optimum pH for the production of hydrogen ranges from 5 to 6; an active control of the pH within the reaction's environment appears to be essential, since pH tends to fall below the correct range due to the formation of VFA during fermentation processes. Lee et al (2002) show how the batch reactors without pH control rapidly decrease the production of hydrogen, due to inhibition by pH.

One of the most recent proposals in the context of the production of H2 in a two-stage reactor, separating the methanogenic phase from the hydrogenic one, is that of Kraemer and Bagley (2005) that reduces by 40% NaOH necessary to control pH through the recirculation of the effluent from the methanogenic stage to the hydrogenic one.

Methanogens' inhibition

Several studies on hydrogen production by fermentation processes, traditionally single-stage, have dealt with the problem of inhibition of methanogens, identified as the main responsible for the rapid consumption of hydrogen. The three most used strategies are:

-heat shock : inoculum is heated to 100°C or higher to inactivate hydrogenotrophic bacteria and concentrate sporigens anaerobic ones;

-pH control: inhibition/inactivation of methanogens through low pH values;

-use of bromoetansulphate (BES): supposed inhibitor of methanogens, it did not provide expected results; too high concentrations required.

Research to maximize hydrogen yields led to identify the use of two stages reactors, with the physical separation of hydrogen-producing bacteria and methanogens; this is a possible fourth way to control the bacterial consortia in the digestors. Still, heat shock and low pH are advised.

Nitrogen blowing

If already in 2000, Mizuno et al. (2000) reported an increase in the production of hydrogen of 68% after the injection with nitrogen, Liu et al . (2006) reported an even higher increase of 88% thanks to the injection of treated biogas (carbon dioxyde- and sulphide-free) at a constant flow rate of 120 ml h – 1.

This phenomenon is explained by the decrease of the partial pressure of hydrogen and the carbon dioxide concentration in the reactor . In fact, these two factors greatly influence the synthesis pathway : high hydrogen partial pressures lead to a greater production of reduced substrates (such as lactate , ethanol , acetone, or alanine) , while high concentrations of carbon dioxide favor the production of fumarate or succinate . Another possible explanation is related to the removal of carbon monoxide from the system, which affects the bacterial metabolism by pushing it from the production of hydrogen to the production of solvents (eg ethanol) .

1.5 Two-stage anaerobic digestion

As already mentioned the two-stage anaerobic digestion is an interesting application of the fermentation process as it allows to obtain, from the two stages, separate production of two types of biogas, one characterized by a high content of methane (second stage) and the 'other characterized by a high content of hydrogen (the first stage).

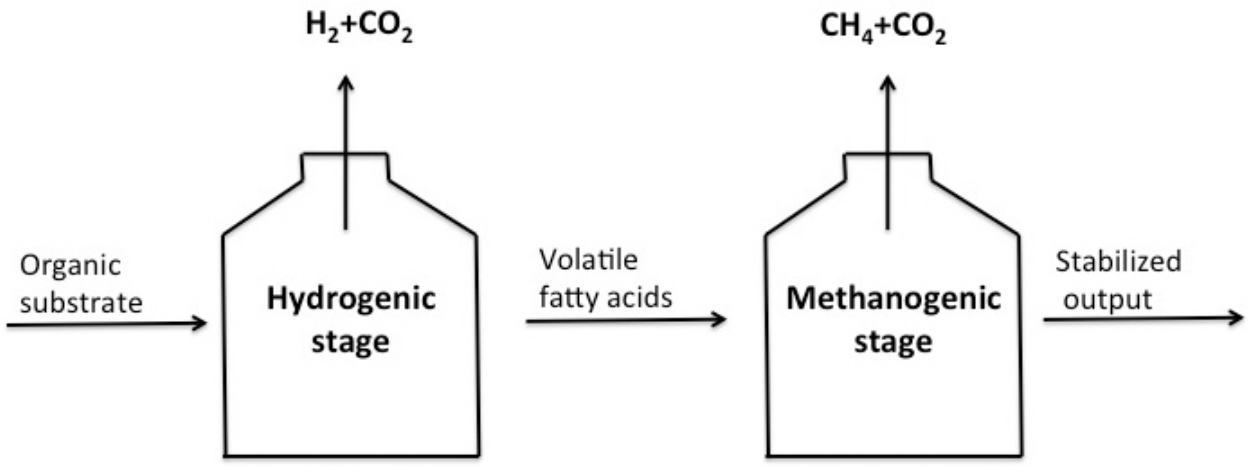

Fig.1.2: two-stage process.

This process is a recent discovery (Kyazze et al., 2007; Liu et al., 2006; Ueno et al., 2007); the separation of hydrolysis/acetogenesis and methanogenesis allows to enhance the single processes, thus leading to higher speed and reaction yields ((Fox and Pohland, 1994).

This system has proven to be particularly reliable and stable for waste with high biodegradable wastes such as fruits and vegetables.

This is due to the fact that the rapid hydrolyzation and acidification which would lead to a lowering of the pH, with accumulation of volatile fatty acids inhibiting the methanogenic biomass, takes place in the first reactor, while preserving the second from this kind of problems(Pavan et al., 2000).

Many other studies proved the feasibility of this method (Cai et al., 2004; Liu et al., 2006), but they are more focused on the optimization of both single stages (Antonopoulou et al., 2008; Venetsaneas et al., 2009).

It is necessary to optimize the entire system to a higher overall energy production. In addition, the mechanisms involved in the two-stage process and the microbial communities have not been investigated and clarified yet, because they are crucial points to a deeper understanding of the process.

The interest for the two stage systems grew in response to some studies that report that, in addition to the production of hydrogen on the first stage, the use of pre-digested material in the second stage maximize the production of methane(Lay et al., 1999).

If the traditional single-stage process lead to generate biogas with a CH4 content of 55-60%, the biogas produced in the second stage may in fact contain up to 80%. Managing separately microbial environments of the two stages allows to optimize the production of each also acting on the different volumetric ratios, in order to take into account the different speeds of individual metabolic phases and avoid choking typical process of a "cascade".

At the end of hydrogenic phase the effluent presents a high content in

VFA, and this represents an ideal substrate for the subsequent methanogenic phase; having separate stages so makes it possible to dose the amount of effluent input, without acting directly on the metabolism of the first stage.

It was also demonstrated that the combined production of H2 and CH4 in two-stage AD has the potential to produce 30% more energy compared to the traditional single-stage process(Liu et al. 2006, Luo et al. 2011).

Furthermore, the mixture of methane and hydrogen has many advantages compared to only methane: it can improve engine efficiency and reduce emissions of CO2 and CO (Akansu et al., 2004).

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2. PREDICTING BIO-HYDROGEN PRODUCTION BY DARK FERMENTATION USING BIOMASS' CHEMICAL COMPOSITION

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Abstract

Bio-hydrogen production throughout dark fermentation (DF) is gaining importance for the production of energy from biomasses. Nevertheless, not all biomasses are suitable to produce H_2 as chemical composition affected this ability. In this work a first attempt to predict H_2 production by DF starting from chemical composition is presented. Different biomasses of different origin were chemically characterized and tested for H_2 productivity in comparison with pure glucose. Results obtained indicated that H_2 production depended, essentially, by soluble sugar and starch content. The successive application of partial least square analysis (PLS) provided an useful tool to predict H_2 production with good predictability.

Key words: Bio-hydrogen; biomasses; dark fermentation; soluble sugar; partial least square regression (PLS).

2.1 Introduction

In the latest years a growing interest towards renewable fuels has been developing. Civilized societies have thus far relied on fossil fuels, whose exhaustibility and environmental impact are already well known, as scientific community and media are insistently pointing out. In this context developing methods allowing safe and eco-friendly energetic provision is necessary and urgent [1]. Hydrogen is mostly used for the synthesis of ammonia (49% of production), oil refinery (37%), methane production (8%), synthesis of other substances (6%) [3]. Only 3% of total hydrogen is involved in direct energy production. A promising application of hydrogen is its utilization in fuel cells [2], with conversion efficiencies to electric power of up to 60%. Moreover hydrogen could be used in new homogeneous charge compression ignition (HCCI) engines (efficiency close to 45%) [4] [5].

Hydrogen can be produced through different pathways: from fossil fuels (steam reforming, partial oxidation, coal regasification and thermal cracking), from water (electrolysis, photolysis, thermolysis, thermochemical processes) and from biomasses (pyrolysis, gasification, microbial conversion). Biological processes are proving to be quite promising for the production of hydrogen, due to their low energetic impact (they take place at room temperature and pressure), and their high environmental sustainability (they can be performed by using waste materials, thus combining the organic waste disposal).

The growing needs for renewable energy sources, alternative to fossil fuels, and for new perspectives for agricultural compartment, determined a major interest for energy chain and for the development of energy farms [6], i.e. farms complementing normal agricultural activities with the production of renewable energy. One of these energy sources is biogas produced by anaerobic digestion (AD) of biomasses [7].

While traditional AD allows the production of a biogas with high concentration of methane, more recent technologies (two-stage AD) allow obtaining two different streams of biogas, rich in

hydrogen and methane, respectively. Bio-methane already has a wide array of applications, while bio-hydrogen, characterized by a high calorific power and virtually no dangerous emissions from combustion [8], is gaining importance at global level for the production of energy. Still, the process of adapting AD to product bio-hydrogen needs deeper studies due to technological limitations. Biomass chemical composition could greatly affect H_2 production but few data are available in literature. For examples, if nitrogen is commonly listed as an important nutrient for bacterial consortia [9], there is still no evidence about the role of proteins in the increase of hydrogen production by dark fermentation [10]. Again, it was reported that lipid hydrolysis leads to the reduction of glycerol, a substrate that bacteria are able to use for growing; in addition the formation of carbohydrates and VFA (volatile fatty acids) (important substrates for hydrolysis) from lipids was reported to be involved in hydrogen production [11].

Biogasification tests have been reported to be useful in determining potential biomethane production of biomasses and in this way they are widely used [12]. On the other hand, there is still much to investigate about bio-hydrogen potential production of different biomasses. Relatively few data are available about bio-hydrogen potential test, although a first tentative was recently reported in literature [13]. In this study, the above reported procedure [13] was re-adapted and potential H_2 production was detected for a group of different organic materials. In addition, complete chemical characterization of the tested biomasses was performed in order to understand how chemical composition affected H_2 production by dark anaerobic digestion.

2.2 Materials and methods

2.2.1 Organic matrices

Seven different organic substrates were used to perform H_2 potential biogasification test: corn silage, malt powder, beet pulp, giant reed (*Arundo donax* L.), olive pomace, rice middlings and glucose. Samples were selected in order to obtain a variability in chemical composition and origin.

All samples were dried at e40°C until constant weight, then milled at passed through a 0.5 mm screen.

2.2.2 Bio-H_2 potential production (BHP)

Startup inoculum was collected from a 10-l lab-scale reactor producing hydrogen under thermophilic conditions (55°C) and at pH value between 5 and 6; the reactor was monitored for several weeks to ensure a stable H_2-gas production [13].

Before the test, the inoculum was shocked in oven at 100°C to inhibit methanogen microorganisms and diluted with water to obtain an optimal volatile fatty acids (VFA) concentration (VFA < 800 mg l^{-1}). H_2 production tests were performed under batch-modality by placing 300 ml of inoculum into 500 ml glass vials, flushed with N_2 and incubated at 55°C. Batch reactors were then fed with 0.3 g of dried biomass. All tests were performed in triplicate.

Volumetric production of biogas was daily monitored through graduated syringes. Hydrogen content was analyzed through a micro gas cromatograph (Micro GC 3000, Agilent Technology, Santa Clara, CA, USA). The test was carried on till biogas production reached a plateau (no further H_2 was produced).

2.2.3 Chemical characterization

Total Solids (TS) and Volatile Solids (VS) contents were evaluated according to standard procedures [14] [15]. Total Kjeldhal Nitrogen (TKN) was detected on fresh material, while organic nitrogen content was used to evaluate the total protein content of samples [16].

Van Soest method [17] was used to evaluate fiber content on dry samples milled at 0.5 mm. NDF (Neutral Detergent Fiber), ADF (Acid Detergent Fiber) and ADL (Acid Detergent Lignine) data, were used to calculate the content of lignin-like fraction, cellulose (ADF-ADL), hemicellulose (NDF-ADF) and soluble cell content (100-cellulose-emicellulose-lignine). Total fats content was determined by Soxhlet extraction with ether [18]. Hall method [19] was used for the determination

of sugar content (TESC, Total Ethanol-Soluble Carbohydrate 80%) while the amyloglucosidase/α-amylase kit method was used for the determination of total starch [20].

In vitro digestibility was measured according to Robinson et al. [21]: dried samples were placed into individual *in vitro* incubation bags (multi-weave polyethylene polyester polymer cloth) and incubated in a DAISYI *in vitro* system (Ankom – Macedon, NY, USA).

The gross energy (GE) was measured using the adiabatic bomb calorimeter IKA 4000 (IKA®-Werke GmbH & Co. KG, Staufen, Germany).

Easily degradable organic matter contents of substrates was measured according to Schievano et al. [12], by detecting the Specific Oxygen Uptake Rate (SOUR), that is the measurement of the oxygen uptake rate during microbial degradation of the organic substrate suspended in a continuously agitated water solution at 37 °C.

2.2.4 Statistical approach

Data set employed to perform statistical study was composed by the chemical and physical parameters characterizing the seven biomasses. A Pearson correlation matrix was performed by using the SPSS statistical software (version 17; SPSS, Chicago, IL). Data were transformed to normality according to the literature [22].

Multiple linear regressions to predict bio-hydrogen potential production (BHP) vs. chemical and physical variables, were detected using the Partial Least Square method (PLS) [23].

The cross-validation "leave-one-out" approach of scaled variables was applied to calculate the goodness of regressions (goodness of fit coefficient-R^2 and goodness of prediction coefficient-R^2cv, respectively). Taking into consideration all variable values the best PLS regression was calculated and the importance of each independent variable (importance coefficient) defined. Then PLS analysis was repeated excluding the variables characterized by the smallest importance coefficient [23]. This procedure was repeated until a final regression model with high regressions coefficients

(R^2 and R^2cv) and the smallest number of variables was achieved. PLS was performed using SCAN software (Minitab Inc., State College, PA).

2.3 Results and discussion

A wide diversity in chemical-physical composition in the organic matrices studied was evideced. Pure glucose, used as reference substrate, as expected showed peculiar characteristics, i.e. VS of 100% TS, soluble sugar of 100% TS, high degradability: OD_{20} of 250 gO_2 kg^{-1}TS *20 h^{-1}. The other substrates showed low variability in VS contents that ranges from 834 g kg^{-1} TS for rice middlings to 950 g kg^{-1} TS TS for giant reed. Rice middlings were the most protein-content biomass analyzed, with a content of raw proteins of 15.9 % TS in opposite to beet pulp that showed the lowest protein content (4.3% TS) (Table 1). Olive pomace showed the highest content of total lipids, with an ethereal extract of 12.7 % TS and the beet pulp the lowest (0.44 % TS). Olive pomace had the highest ADL content (38% TS), while malt powder the lowest one (3.5 % TS). The highest starch content was found, such as expected, in the malt powder (67.7% TS), while the lowest content was for the rice middlings (0.46 % TS); rice middlings and olive pomace showed null content of soluble sugars, while corn silage showed a content of this fraction of 14% TS.
The energy content varied from 17,087 kJ $kgTS^{-1}$ for beet pulp to 23,871 kJ $kgTS^{-1}$ for olive pomace, while *in vitro* digestibility ranged from a minimum of 34.7% TS for giant reed to a maximum of 94.2% TS, for beet pulp. SOUR test (OD_{20}) results indicated a degradability of substrates that ranged from 53.4 g O_2 kg^{-1} TS * $20h^{-1}$ for giant reed to 128 g O_2 kg^{-1} TS * 20 h^{-1} for malt powder.
Pure glucose produced 186±10 NlH_2 kg^{-1} TS, i.e. 1.37 mol_{H2} $mol^{-1}_{glucose}$; this result, although not as high as expected, was in line with data reported by Tenca et al. [13].
The BHP test indicated lower H_2 production for all matrices when compared with glucose H_2-production. As expected, highest hydrogen yields were obtained for biomasses rich in soluble and more readily available compounds such as soluble sugars and starch (corn silage and giant reed).

On the other hand, matrices with high percentages of recalcitrant components, such as lignin-like fraction, produced low quantity of H_2 (i.e. olive pomace) (see Figure 1).

A series of Pearson correlations between BHP and different chemical components (both by themselves and combined) were performed on normalized data (Table 2). Pearson analysis showed strong negative correlations of H_2 production vs. recalcitrant compounds, especially with the less digestible portions of fibers, i.e. ADL ($r=-0.96$, $p < 0.01$) and the combination of ADF plus ADL ($r=-0.80$; $p < 0.05$).

A significant positive correlation between H_2 production and soluble sugars ($r=0.80$, $p<0.05$) was found. On the other hand, no correlation was found between starch and H_2 production. Differently, when considering the sum of soluble sugars and starch, the positive correlation with H_2 production was stronger than for soluble sugars alone ($r=0.87$, $p<0.05$).

No significant correlations were found for other fractions of the organic matter, such as proteins and lipids, NDF, ADF, cellulose and hemicellulose, confirming that H_2 production pathways are strongly linked to soluble sugars. This may be caused by two factors: a) the chemical component is hardly available to fermentative microflora, because its hydrolysis has too slow kinetics; b) the chemical component is quickly hydrolyzed and available to fermentation, but do not follow a metabolic pathway that drives to H_2 production.

To look into this hypothesis, OD_{20} and digestibility, which are parameters that indicate the short-term availability of organic matter to biodegradation, didn't show significant correlation with H_2. This confirm that not all the easily-hydrolysable fractions of organic matter (e.g. proteins), that are fermented during acidification process, can be related to methabolic pathways driving to H_2 release.

At the same time, the H_2 production measured for the giant reed was interesting and unexpected, as soon as typically the low digestibility of this plant (see the relatively low OD_{20}, Table 1) should have driven to low H_2 yield,. Nevertheless, the relatively high soluble sugar content (Table 1) allowed obtaining H_2 production as high as other matrices, i.e. malt powder and rice middlings, for which high H_2 production was expected.

Table 2.1. Substrates characterization.

Sample	–	*Corn silage*	*Malt powder*	*Beet pulp*	*Olive pomace*	*Rice middlings*	*G. cane*	*Glucose*
Hydrogen production	$NLH_2\ kg^{-1}TS$	106±23	107±13	69.9±3.1	48.7±8.9	96.6±5.9	102±10	186±10
Organic matter	$gTS\ kg^{-1}TS$	914±0	852±0	898±0	940±0	834±0	950±0	1000
Raw proteins	$gTS\ kg^{-1}TS$	150±1	61.7±0.1	43.2±0.1	131±0	159±0	59.8±3.2	0
Ethereal extract	$gTS\ kg^{-1}TS$	18.2±0.1	79.8±0.1	4.40±0.02	127±0	32.5±0.2	38±0	0
NDF	$gTS\ kg^{-1}TS$	522±1	360±2	446±1	669±2	534±2	826±3	0
ADF	$gTS\ kg^{-1}TS$	301±1	173±1	254±1	584±2	375±2	558±3	0
ADL	$gTS\ kg^{-1}TS$	57.6±0.3	35±0	120±1	383±1	131±1	128±1	0
Starch	$gTS\ kg^{-1}TS$	151±0	677±3	30.3±0	8±0.01	4.6±0	28.5±0.1	0
Soluble sugars	$gTS\ kg^{-1}TS$	140±1	1.5±0	1.72±0.03	0	0	100±0	1000
Energy content	kJ/kgTS	19,273±185	17,207±219	17,087±220	23,871±304	17,937±195	18,732±200	14,222±114
Digestibility	$gTS\ kg^{-1}TS$	771±2	393±1	942±4	362±2	524±2	347±1	100
OD_{20}	$gO_2\ kg^{-1}TS\ *20\ h^{-1}$	102±5	128±5	94±4	72±2	95.4±2.2	53.4±1.7	250

The correlation found confirmed that H_2 production is strictly correlated to substrate composition in terms starch and soluble sugar contents, as previous literature often highlighted [11]. In particular, Akutsu et al. [24] proved the positive influence of the presence of starch in biomasses on H_2 production. Therefore, accessible path to produce bio-hydrogen is the utilization of carbohydrate-rich biomass, as shown by Noike et Mizuno [25] and Yu et al. [26].

The results here obtained indicate that H_2 production should be driven by soluble sugar and starch contents and that the quantification of these two chemical parameters can be used to predict H_2 production.

Doing so, a partial least square analysis (PLS) was performed on normalized data to detect which chemical components, among those measured, influenced hydrogen production and their relevance. Multiple PLS resulted in a linear regression model able to predict H_2 production according to the following equation:

$$BHP = 110.24 - 20.04\ arcsin\sqrt{ADL} + 11.4 arcsin\sqrt{soluble\ sugars} + 0.3749*(starch+soluble\ sugars)$$

In which BHP is expressed as Nl kg^{-1}TS and ADL, soluble sugar and starch as % of TS.

Equation showed good regression coefficient (R^2=0.91, $p<0.05$) and high predictability (R^2cv=0.84, $p<0.05$), such as the comparison between experimental and calculated data confirmed (Figure 1).

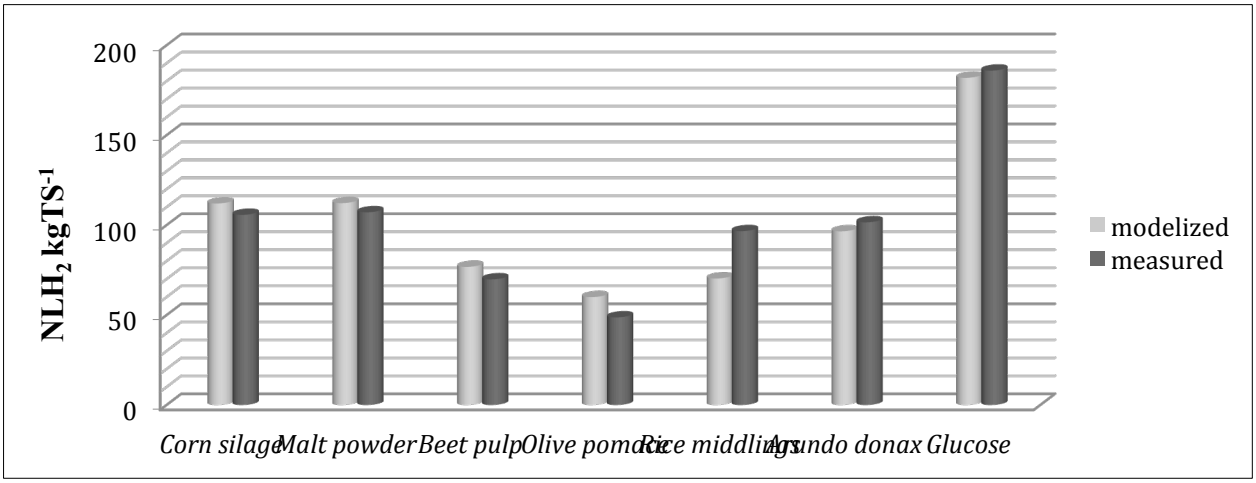

Figure 2.1: Comparison between modelized and measured biohydrogen production.

2.4 Conclusions

This work indicates that H_2 productivity depends, essentially, on the presence of consistent fractions of soluble sugar and starch in the organic matter. The application of partial least square analysis(PLS) provided an useful tool to predict H_2 productivity, with good predictability. Further efforts in this pathway should provide wider information on this topic.

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2.6 Addendum

A new set of samples was used in an extension of the previous study.

Ten samples of different origin (industrial, agricultural and urban wastes, as well as crops) underwent the same procedure shown above.

Samples included sludge from a depuration plant, sugar blueberry extract, citrus pellet, a mixture of vegetables (potatoes, peppers, onions) from a food factory; from a waste treatment plant compost and a mixture of urban and green wastes were obtained; last, from the agricultural compartment milled cob, manure, marc and ryegrass were used.

These matrices were analyzed as seen above; digestibility and energy content proved to be not interesting parameters and were therefore not measured. Results are shown in table 2.2

Tab.2.2: chemical characterizations.

Sample		*Sludge*	*Blueberry extract*	*Citrus pellet*	*Solid urban wastes mixture*	*Food industry waste*	*Compost*	*Milled cob*	*Manure*	*Marc*	*Ryegrass*
Hydrogen production	NLH_2 kg^{-1} TS	7.98±0.2	123±3.2	104±2	7.98±0.8	31.8±1.4	11.1±0.6	60±2.1	11.7±0.52	12.7±0.43	11±0.44
Organic matter	$gTSkg^{-1}$TS	934±11.3	967±2.9	934±10.4	751±0.9	930±1.77	763±3.09	986±4.7	805±14.9	916±12.6	901±1.04
Raw proteins	$gTSkg^{-1}$TS	30.3±0.6	0.1	68±1.31	147±9.87	153±2.54	134±1.34	66.2±0,5	114±1.1	110±0.93	73.1±2.66
Ethereal extract	$gTSkg^{-1}$TS	810±7.41	54.6±0.43	14.9±0.8	167±1.41	124±2.3	160±0.92	110±0.81	101±2	135±1.1	208±3.07
NDF	$gTSkg^{-1}$TS	0	0	218±2.4	443±10.3	503±11.4	436±5.61	131±1.11	624±5.47	645±1.07	472±4.01
ADF	$gTSkg^{-1}$TS	0	0	173±1.03	305±1.92	389±21	398±9.08	46±0.97	434±21.3	604±33.1	344±13.5
ADL	$gTSkg^{-1}$TS	0	0	140±2.32	122±4.41	227±18.7	265±11.3	9±0.03	241±14.5	407±20.4	66.1±0.8
Starch	$gTSkg^{-1}$TS	17.4±0.9	0	87.5±1.44	73.7±0.88	80.1±3.24	59.9±0.85	73.11±3.81	22.1±0.77	99.2±1.46	58.9±1.3
Soluble sugars	$gTSkg^{-1}$TS	0	945±10.5	285±10	39.9±0.97	9.52±0.55	36.6±0.39	57.43±2.77	154±1.19	21.1±0.99	200±3.98
OD20	gO_2kg^{-1}TS*20h	135±1.92	308±5.06	232±8.54	149±1.01	191±0.9	223±15.4	326±9.04	55.3±2.32	92±2.35	134±5.56

The application of the same model as the first data set was not successful. A quicker statistical analysis was performed. A Pearson series of correlation was carried out; in table 2.3 correlations between chemical parameters and hydrogen production are shown.

Tab. 2.3: Pearson's correlations

	R	R^2
Organic matter	0,62	0,38
Raw proteins	-0,7	0,49
Ethereal extract	-0,45	0,20
NDF	-0,65	0,42
ADF	-0,62	0,38
ADL	-0,68	0,46
Starch	-0,48	0,23
Soluble sugars	0,89	0,79
OD20	0,59	0,35

Due to the higher number of samples and their wider differences, correlations are not as immediate. Still, a strong positive correlation ($R=0.89$, $p<0.01$) was detected between hydrogen production and soluble sugars. A negative correlation is seen with raw proteins ($R=-0.7$, $p<0.05$) and ADL ($R=0.68$, $p<0.05$), thus confirming the dependence of hydrogen production on the abundance of immediately available compounds such as soluble sugars, while the most recalcitrant ones proved again to provide a negative influence.

3. TWO-STAGE INSTEAD OF ONE-STAGE ANAEROBIC DIGESTION CAN REALLY INCREASE ENERGY RECOVERI FROM BIOMASS

Submitted to Applied Energy

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Abstract

The supremacy of two-stage on traditional one-stage anaerobic digestion (AD, in terms of overall energy recovery (ER) from biomass has often been proved. However, the process conditions ensuring this result, as well as the reasons for higher efficiency, have always been unclear. In this work, a new standardized approach is proposed: optimization at lab-scale of both hydrogen and methane generation processes allowed comparing the maximum potential ER of both two-stage (as H_2+CH_4) and one-stage AD (as CH_4). Relatively high biohydrogen yields were obtained testing four different organic substrates (ER of 1 – 1.6 MJ $kg^{-1}_{VS\text{-}added}$). Biomethane generation resulted in ER in the range 9 – 19 MJ $kg^{-1}_{VS\text{-}added}$., similarly for two-stage and one-stage systems. The overall ER resulted significantly higher (8 – 43%) for the two-stage in the large majority of experimental conditions and never significantly lower. These preliminary results should drive further research to better understand the conditions that can drive the two-stage AD to higher performance.

Keywords: Two-stage anaerobic digestion, biohydrogen, biomethane, biomass, bioenergy

3.1 Introduction

The two-stage anaerobic digestion (AD) process has often been reported as a viable way to produce bio-hydrogen and bio-methane from a wide range of organic materials [1,2]. In the last decade, several studies were published on this topic and many authors reported different applications of the two-stage AD, with different organic substrates and different process designs [3,4,5].

Generally, the phase separation of hydrolysis/fermentation from methanogenesis in different reaction environments has been proposed as a strategy to increase overall process performances, in terms of stability, degradation efficiencies in both fermentation and methanogenesis phases and thereby in terms of overall energy recovery (ER) from biomass [6]. A controlled acidogenic fermentation, that allow efficient bio-hydrogen production, has been thought the best pathway to pre-treat raw biomass to enhance methanogenic process.

According to various authors, efficient bio-hydrogen production and volatile fatty acids (VFA) liberation in the liquid during acidogenic phase would at the same time ensure energy recovery as H_2 and favor CH_4 production from VFA in the methanogenic reactor [7].

On the other hand, literature has seldom given general and exhaustive explanations to this thesis, often limiting efforts on particular case studies, with particular substrate types and operational conditions.

In particular, few studies took into account the overall potential ER of two-stage AD, compared to single-stage AD, focusing on the reasons and the conditions for actual enhancement of ER by phase separation. The most important contributions to this topic came from Liu et al. [8], Pakarinen et al. [9] and Luo et al. [6], that demonstrated the supremacy (from 20 to 60% higher ER) of two-stage AD at both thermophilic and mesophilic conditions. The reasons for success of two-stage system were associated, generally, to process advantages, as higher efficiency in converting VFAs into methane in the second stage [8]. Pakarinen et al. [9] obtained high advantage from two-stage AD and found significantly higher hydrolysis efficiency after the hydrogen production step, with increased soluble organic matter and VFA production through fermentation, allowing higher

productivity in the methanogenic phase. Luo et al. [6] were more precise and associated the higher ER of the two-stage to higher performances in the second-stage (methanogenesis) in degradation kinetics and to the effect of minimizing the loss of relatively ‘‘fresh feed’’ out of the reactor due to ‘‘short-circuiting’’, occurring in single-stage fully mixed reactors.

More recently, Schievano et al. [10] observed two-stage *vs* single-stage AD in thermophilic continuously stirred tank reactors (CSTR) fed with a mix of fruit/vegetable waste and swine manure, focusing on the overall ER and on biological process efficiencies. In this case, equal ER resulted from the two AD systems, even if the methanogenic reactor in the two-stage system showed residual un-degraded organic compounds (VFA were 10 times higher than in the single-stage reactor) and thereby an unexpressed potential [10]. This means that, in this case, if the methanogenesis in the two-stage wasn’t slightly inhibited, the two-stage would have shown higher ER, as compared to the single-stage.

In this work, a new approach in investigating this topic is proposed. Both bio-hydrogen and bio-methane productions should be always optimized to compare the two AD systems. For this reason, optimization of bio-hydrogen production process was carried out for four different organic mixtures and the biochemical methane potential (BMP) standard tests were used to obtain optimized ER from methanogenesis.

3.2 Materials and Methods

To verify the energy recovery two-stage and single-stage AD were simulated in lab-scale fermenters. The test was run on four different organic mixtures of biomass, diluted with liquid swine manure (SM) to the desired organic matter concentration, measured as volatile solids (VS) per g of wet weight (ww). Indeed, SM is a very common liquid material used in biogas plants and provides both nutrients and buffer capacity to AD environments. In previous studies dealing with optimization of anaerobic dark fermentation, SM was already used as co-substrate to efficiently produce bio-hydrogen [11]. The feeding substrates were 4 organic materials, usually available in

full-scale agricultural AD facilities: a) maize silage (MS), b) waste rice flour (RF), c) olive pomace (OP) and d) waste fruit/vegetable (FV).

The first-stage was run (as reported by Tenca et al. [11]) in semi-continuous reactors, fed twice a day; the optimized H_2 production were selected by varying the feeding conditions in two variables: i) organic matter concentration (OMC) and ii)hydraulic retention time (HRT). The pre-digested materials, produced in optimized conditions, underwent the methanogenic phase (2^{nd} stage), i.e. incubated in batch reactors optimized for methanogenesis. The single-stage AD was run in parallel in batch reactors fed with the untreated organic mixtures. The energy recovered from the double-stage (H_2+CH_4) and the single-stage (CH_4) AD systems were compared to look for possible increase in productivity in the double-stage concept.

3.2.1 Hydrogenic process optimization (1^{st} stage)

The hydrogenic phase of two-stage AD system was run in semi-continuously operated reactors of 500 mL capacity, fed 2-times a day, in thermophilic conditions (55±1ºC), as reported in detail in a recent work by Tenca et al. [11]. A Box-Wilson central composite design (CCD) [12] was applied to study the effect of two operating parameters (the controllable factors: OMC and HRT) on biohydrogen production (the experimental response), and therefore to find the optimal region in which to operate 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 -√2 and +√2 from the center of the design. As a result, OMC and HRT were investigated at five levels, coded as (-√2, -1, 0, +1, +√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, hereafter called experimental conditions (EC), corresponding to nine combinations of OMC with HRT values. Each treatment consisted of three replicated assays, except for the centerpoint EC, which was replicated six times. For all substrates, except for FV, the selected ranges for factors were 25 – 65 g_{VS} kg^{-1}_{ww} and 1 – 4 d for OMC and HRT, respectively, with a design centerpoint of [45 g_{VS} kg^{-1}_{ww}; 2.5 d]. The resulting investigated range for OLR was from 8.9 to 45 g_{VS} $L^{-1}_{dig.}$ d^{-1}. According to results of experiments conducted in previous work with a similar substrate [11], for FV the selected factors ranges were 27 – 72 g_{VS} kg^{-1}_{ww} and 1 – 3 d for OMC and HRT, respectively, centerpoint of the design being [50 g_{VS} kg^{-1}_{ww}; 2 d]. The corresponding range for the organic loading rate is approximately from 12.4 to 52.8 g_{VS} L^{-1} d^{-1}. All the coded levels and corresponding values of operating variables considered in the experimental design are summarized in Table 3.1.

Table 3.1 - Experimental design of the first stage (hydrogenic) loading in two variables (OMC *vs* HRT) and theoretical organic loading rates (OLR) for the organic mixtures studied.

Sample	EC	OMC $g_{VS}\ kg^{-1}_{ww}$		HRT d		OLR $kg_{VS}\ L^{-1}_{1}\ d^{-}$
MS + SM	**1**	0	45.0	-√2	1	45.0
	2	-1	31.0	-1	1.5	20.7
	3	1	59.0	-1	1.5	39.3
	4	-√2	25.2	0	2.5	10.1
	5	0	45.0	0	2.5	18.0
	6	√2	64.8	0	2.5	25.9
	7	-1	31.0	1	3.5	8.9
	8	1	59.0	1	3.5	16.9
	9	0	45.0	√2	4	11.3
RF + SM	**1**	0	45.0	-√2	1	45.0
	2	-1	31.0	-1	1.5	20.7
	3	1	59.0	-1	1.5	39.3
	4	-√2	25.2	0	2.5	10.1
	5	0	45.0	0	2.5	18.0
	6	√2	64.8	0	2.5	25.9
	7	-1	31.0	1	3.5	8.9
	8	1	59.0	1	3.5	16.9
	9	0	45.0	√2	4	11.3
OP + SM	**1**	0	45.0	-√2	1	45.0
	2	-1	31.0	-1	1.5	20.7
	3	1	59.0	-1	1.5	39.3
	4	-√2	25.2	0	2.5	10.1
	5	0	45.0	0	2.5	18.0
	6	√2	64.8	0	2.5	25.9
	7	-1	31.0	1	3.5	8.9
	8	1	59.0	1	3.5	16.9
	9	0	45.0	√2	4	11.3
FV + SM	**1**	0	50.0	-√2	1	50.0
	2	-1	34.0	-1	1.25	27.2
	3	1	66.0	-1	1.25	52.8
	4	-√2	27.4	0	2	13.7
	5	0	50.0	0	2	25.0
	6	√2	72.6	0	2	36.3
	7	-1	34.0	1	2.75	12.4
	8	1	66.0	1	2.75	24.0
	9	0	50.0	√2	3	16.7

All reactors were initially inoculated with a digested material collected in a 10 L laboratory-scale reactor, digesting a mixture of the four organic substrates used in this study. The digester had

been continuously operating under thermophilic conditions (55 °C) for approximately 20 days, prior to the beginning of this study, showing a stable production of biohydrogen. The TS and VS concentrations and the pH of the inoculum resulted in 36.1 ± 4.3 g kg^{-1}_{ww}, 29.4 ± 3.6 g kg^{-1}_{ww} and 5.65 ± 0.23, respectively.

The test was prolonged for almost 10 – 15 days, till the production of biogas conditions was stable. The last 5 days of stable production were taken into account for data elaboration and for sampling the pre-digested materials. Biohydrogen production was calculated from volume measurements of gas accumulated in sample bags and by measuring its hydrogen content.

3.2.2 Methanogenic process (2nd stage and single-stage)

Optimized methanogenic process was applied to raw materials (simulating one-stage process) and to treated materials (simulating the second stage process). Only the most productive EC were chosen, i.e. those reaching hydrogen yield (NL_{H2} kg^{-1}_{VS}) above 30% of the most productive EC for each biomass type. Treated materials sampled 4 different times from the hydrogenic reactors at steady state and mixed together in one single sample.

The optimized methanogenic process was performed in batch thermophilic reactors of 500 mL capacity. This test was adapted from standard procedures of bio-chemical methane potential (BMP) [13], which ensures optimized conditions for methanogenic activity. In brief, 300 mL of operating volume were used, with approximately 200 mL of headspace in the batch. The samples of treated (for double-stage AD) or raw (for single stage AD) substrate were added to inoculum at a ratio of 1:4 (substrate:inoculum) on wet weight base. The samples were homogenized and used fresh (without drying) in the methanogenic test, to avoid VFA evaporation. The inoculum was a digested slurry (around 45 g_{TS} kg^{-1}_{ww}) sampled in a thermophilic AD facility operating in the agricultural context around Milan, Italy. The digestate was filtered through a stainless steel sieve (US Mesh No. 10, sieve opening of 2.0 mm) and incubated for 15 days before the beginning of the test. After sample addition, the batch reactors were sealed with teflon hermetic

caps, flushed with a N_2 atmosphere and monitored for biogas production (by withdrawing extra-pressure gas with a tight syringe) till plateau was reached, approximately after 60 days. Biomethane production was calculated from volume measurements of gas (withdrawn by tight syringe till equilibrium pressure) and by measuring its methane content.

3.2.3 Measurements and analytical methods

Feeding substrates and treated substrates were characterized in terms of TS and VS (only for feeding substrates), chemical oxygen demand (COD), pH, total volatile fatty acids (TVFA) and total alkalinity (TA) content, according to Standard Methods [14]. All analyses were performed on 4 different samples and the values were expressed as average and standard deviation.

Biogas composition was determined, considering only H_2, CH_4 and CO_2, 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. Carbon dioxide content was analyzed 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 those of Molesieve/5A and Plot U columns were maintained at 100 and 55 °C, respectively.

3.2.4 Total energy recovery calculation

Biohydrogen production rate 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 $L_{H2}\ kg^{-1}_{VS\_added}$. Cumulated biomethane production measured on each batch reactor was also expressed on kg_{VS_added} to the AD system as a whole, i.e. the kgVS added to the hydrogenic stage per each kg of untreated substrate loaded, both in the double- and in the single-stage AD systems.

The biohydrogen and biomethane yields (as $L_{H2/CH4}\,kg^{-1}{}_{VS\_added}$) were transformed into total energy recovery (ER, as $MJ\,kg^{-1}{}_{VS\_added}$), by considering H_2 and CH_4 superior heat of combustion (12.74 MJ/Sm^3 and 35.16 MJ/Sm^3, respectively). The sum of the ER from H_2 and CH_4 represented the total ER from the two-stage AD system, to be compared to the ER obtained by the sole ER of the methane produced in the single-stage AD.

3.3 Results

3.3.1 Biohydrogen productions and yields

All EC were plotted in a chart (Figure 1a) to visualize the experimental set and the variability (as standard deviation) of OMC, during the feeding period. Additionally, the OLRs imposed to the reactors were plotted in Figure1b, to observe that, for the same biomass, each different EC corresponds to a different OLR.

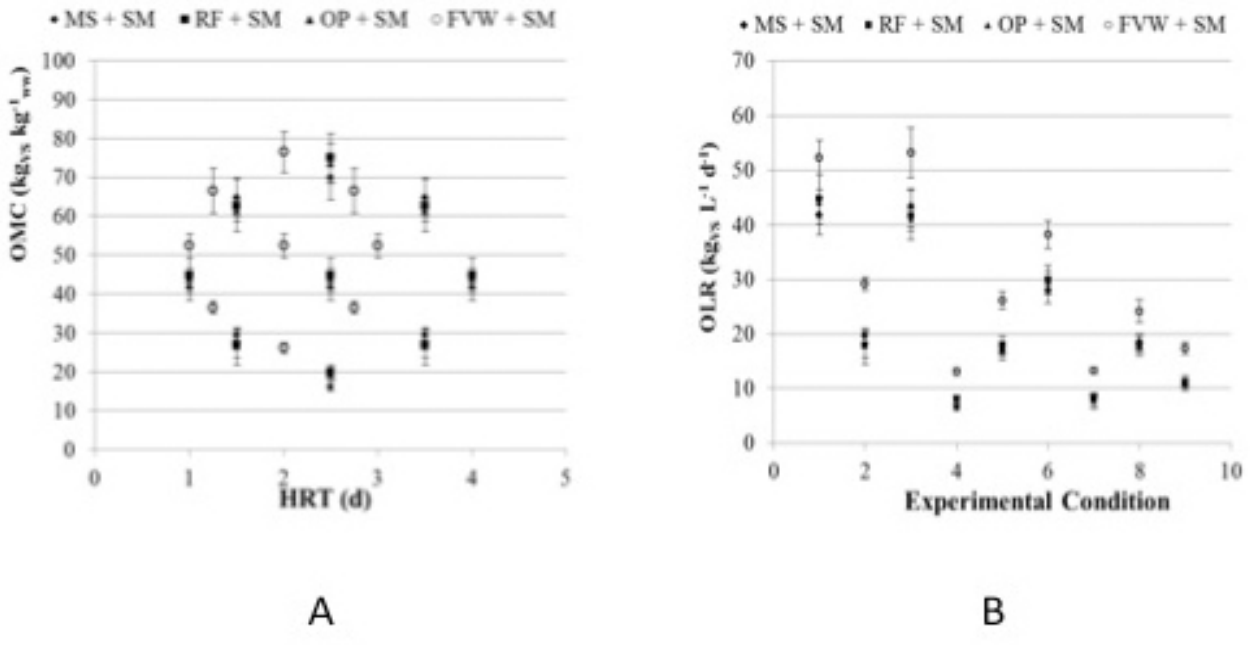

Figure 3.1 a. Experimental conditions (EC) composed by central composite design on the variables organic matter concentration (OMC) and hydraulic retention time (HRT). OMC is

reported with standard deviation on triple samples. **b.** Organic loading rate (OLR) corresponding to each EC.

Biohydrogen productions obtained in all the EC considered in the study are summarized in Table 3.2.

Table 3.2 - Hydrogen and methane productions (as$Sdm^3\ L^{-1}_{dig.}\ d^{-1}$), yields (per $kg_{VS\text{-}added}$) and composition of biogas (as % v/v) produced by the two- and one- stage AD systems.

Mixture	EC	Two-stage ER					One-Stage ER	
		1st stage (H_2)			2nd stage (CH_4)			
		$Sdm^3\ L^{-1}_{dig.}\ d^{-1}$	$Sdm^3\ kg^{-1}_{VS\text{-}added}$	H_2:CH_4:CO_2 %v/v in biogas	$Sdm^3\ kg^{-1}_{VS\text{-}added}$	H_2:CH_4:CO_2 %v/v in biogas	$Sdm^3\ kg^{-1}_{VS\text{-}added}$	H_2:CH_4:CO_2 %v/v in biogas
MS + SM	**1**	3.66 ± 0.34	87.8 ± 8.2	49.6 : 0 : 50.4	380 ± 25	0.2 : 69.9 : 29.9	431 ± 5	0.4 : 69.2 : 30.4
	2	2.37 ± 0.11	120.9 ± 5.6	36.1 : 0 : 63.9	304 ± 18	0.2 : 71.3 : 28.5	403 ± 37	0.3 : 67.2 : 32.5
	3	1.84 ± 0.47	42.6 ± 10.9	33.4 : 0 : 66.6	330 ± 97	0.2 : 67.0 : 32.8	379 ± 24	0.5 : 66.4 : 33.1
	4	0.08 ± 0.02	12.5 ± 3.1	7.4 : 16.2 : 76.3	-*	-	-	-
	5	0.97 ± 0.13	58.2 ± 7.8	42.7 : 0.1 : 57.2	406 ± 95	0.3 : 68.2 : 31.5	431 ± 5	0.4 : 69.2 : 30.4
	6	0.62 ± 0.19	22.2 ± 6.8	35.0 : 0.8 : 64.2	-	-	-	-
	7	0.84 ± 0.14	100.0 ± 16.7	41.8 : 0 : 58.2	504 ± 11	0.1 : 63.7 : 36.2	403 ± 37	0.3 : 67.2 : 32.5
	8	0.85 ± 0.04	46.0 ± 2.2	41.3 : 0 : 58.7	414 ± 4	0.2 : 68.3 : 31.5	380 ± 24	0.5 : 66.4 : 33.1
	9	1.02 ± 0.14	97.9 ± 13.4	43.9 : 0 : 56.1	432 ± 15	0.3 : 57.4 : 42.3	431 ± 5	0.4 : 69.2 : 30.4
RF + SM	**1**	5.74 ± 0.55	128.3 ± 12.3	43.4 : 0 : 56.6	253 ± 18	0.2 : 73.6 : 26.2	295 ± 16	0.4 : 64.9 : 34.7
	2	0.37 ± 0.07	20.5 ± 3.9	6.9 : 10.6 : 82.5	-	-	-	-
	3	5.29 ± 0.63	126.6 ± 15.1	38.7 : 0.1 : 61.2	246 ± 14	0.2 : 71.2 : 28.5	280 ± 11	0.4 : 66.0 : 33.6
	4	0.05 ± 0.03	6.3 ± 3.8	2.7 : 18.7 : 78.6	-	-	-	-
	5	2.08 ± 0.09	116.2 ± 5.0	43.3 : 0.1 : 56.6	305 ± 34	0.1 : 70.7 : 29.3	295 ± 16	0.4 : 64.9 : 34.7
	6	1.58 ± 1.16	52.7 ± 38.7	40.5 : 0.7 : 58.9	316 ± 3	0 : 67.9 : 32.1	271 ± 15	0.2 : 65.7 : 34.2
	7	0.04 ± 0.01	5.2 ± 1.3	4.1 : 19.9 : 76.0	-	-	-	-
	8	1.73 ± 0.20	96.6 ± 11.2	40.9 : 0.3 : 58.8	240 ± 10	0.3 : 71.9 : 27.8	280 ± 11	0.4 : 66.0 : 33.6
	9	1.23 ± 0.09	109.9 ± 8.0	40.2 : 0.3 : 59.5	240 ± 21	0.1 : 76.7 : 23.2	295 ± 16	0.4 : 64.9 : 34.7
PO + SM	**1**	0.02 ± 0.01	0.5 ± 0.2	2.2 : 10.3 : 87.5	-	-	-	-
	2	0.23 ± 0.04	13.0 ± 2.3	1.3 : 13.9 : 84.8	-	-	-	-
	3	0.02 ± 0.01	0.5 ± 0.0	11.1 : 1.1 : 87.8	-	-	-	-
	4	0.01 ± 0.01	1.3 ± 0.0	0.9 : 35.0 : 64.1	-	-	-	-
	5	0.00 ± 0.01	0.1 ± 0.0	0.3 : 33.4 : 66.3	-	-	-	-
	6	0.03 ± 0.01	1.0 ± 0.3	3.3 : 7.4 : 89.4	-	-	-	-
	7	0.02 ± 0.01	2.6 ± 1.3	4.7 : 13.4 : 81.8	-	-	-	-
	8	0.01 ± 0.01	0.6 ± 0.0	2.9 : 13.6 : 83.5	-	-	-	-
	9	0.01 ± 0.01	0.9 ± 0.0	2.1 : 10.8 : 87.1	-	-	-	-
FV + SM	**1**	1.38 ± 0.46	26.4 ± 8.8	18.4 : 0.1 : 81.5	-	-	-	-
	2	0.02 ± 0.01	0.7 ± 0.3	0.3 : 18.0 : 81.7	-	-	-	-
	3	1.74 ± 0.93	32.7 ± 17.5	29.7 : 0 : 70.2	-	-	-	-
	4	0.08 ± 0.02	6.1 ± 1.5	2.3 : 17.5 : 80.2	-	-	-	-
	5	3.24 ± 0.57	123.8 ± 21.8	48.4 : 0 : 51.6	373 ± 11	0.2 : 56.3 : 43.5	293 ± 32	0.2 : 56.1 : 43.8
	6	1.48 ± 0.53	38.7 ± 13.9	21.3 : 0 : 78.7	345 ± 32	0.1 : 56.7 : 43.3	292 ± 6	0.2 : 56.1 : 43.8
	7	0.21 ± 0.23	15.8 ± 17.3	17.5 : 5.6 : 76.8	-	-	-	-
	8	2.39 ± 0.13	98.8 ± 5.4	45.6 : 0 : 54.4	347 ± 18	0.2 : 59.4 : 40.4	278 ± 14	0.1 : 58.0 : 41.9
	9	2.06 ± 0.46	118.1 ± 26.4	37.7 : 0 : 62.3	328 ± 28	0.2 : 54.9 : 44.9	281 ± 31	0.2 : 56.1 : 43.8

* Methane production was not considered for EC where H_2 yields resulted lower than 30% of the maximum H_2 yield

Biohydrogen production rates (per unit of digester volume) resulted higher than 5 $Sdm^3 L^{-1}_{dig.}d^{-1}$ for RF, in EC1 and EC3; contrarily, almost no hydrogen was produced for OP, in every EC. MS and FV reached as best 3.66 ± 0.34 and 3.24 ± 0.57 $Sdm^3 L^{-1}_{dig.}d^{-1}$, respectively. For all substrates, low or no biohydrogen production was obtained in all the assays fed with a substrate having VS concentration below 30 $g_{VS}\ kg^{-1}_{ww}$ (experimental condition 4). Hydrogen concentration in biogas was in the range 30 – 50% v/v for the most productive EC, while lower concentrations were found for the less productive EC (Table 3.2).

The best biohydrogen yields (120 ± 6, 128 ± 12 and 124 ± 22 $NL_{H2}\ kg^{-1}_{VS\text{-}added}$) were obtained at EC2, EC1 and EC5 for MS, RF and FV, respectively (Table 2). They resulted almost equivalent, even if RF showed similar yields also in many other EC (EC3, EC5, EC8 and EC9), while MS and FV only in two EC (EC7/ EC9 for MS and EC8/EC9 for FV). On the other hand, hydrogen yield didn't achieve at least 30% of the maximum value obtained for each biomass in EC4/EC6, EC2/EC4/EC7 and EC1/EC2/EC3/EC7 for MS, RF and FV, respectively (Table 2). Interestingly, the best H_2 productions were always obtained were low methane content (< 1% v/v) was present in the biogas (Table 2), confirming that limiting conditions for hydrogen consumers is a key to optimized bio-hydrogen generation. Both productions and yields were plotted as color-gradient charts (Figure 2), which help in focusing the optimized feed conditions for bio-hydrogen production.

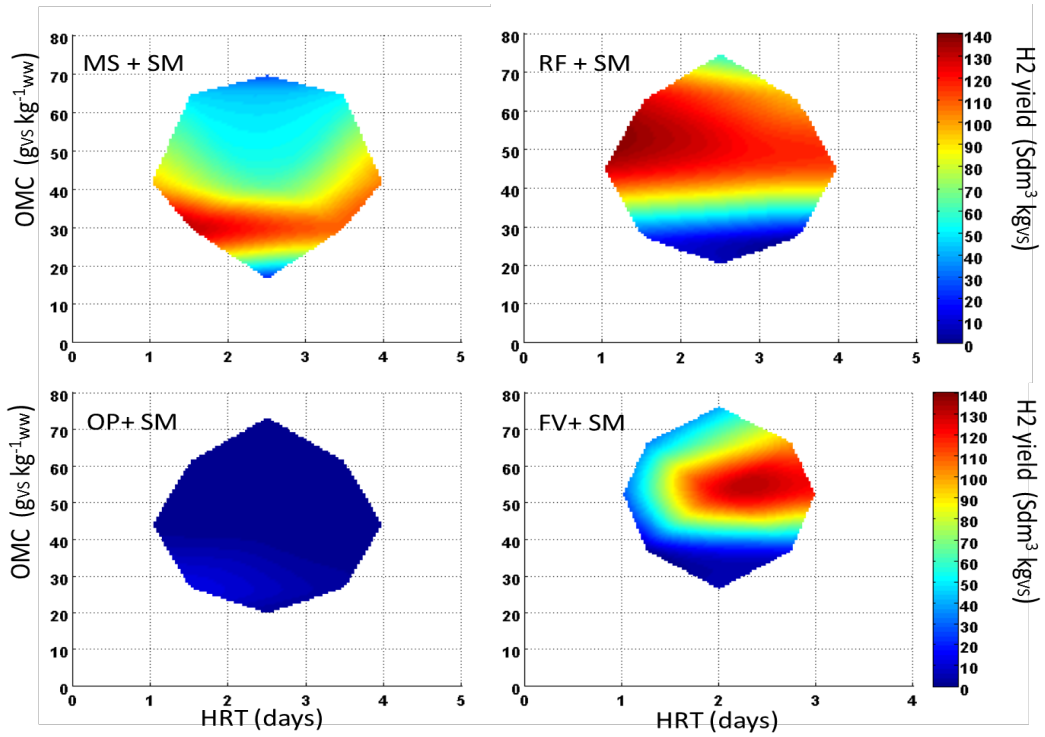

Figure 3.2. Interpolation (color-gradient) of H_2 yield within the considered areas of EC.

3.3.2 Chemical characterization of raw and treated materials

Table 3.3 - Characterization of the raw organic mixtures fed in the 9 points of the experimental design

Sample	EC	TS		OMC		COD		pH in	VFA	Alk	VFA/Alk
		g kg^{-1}_{ww}		g_{VS} kg^{-1}_{ww}		g kg^{-1}_{ww}			g_{l} kg^{-1}_{ww}	g kg^{-1}_{ww}	
MS + SM	**1**	47.1 ±	4.9	41.7 ±	3.4	80.2 ±	7.5	5.86	2.68	11.7	0.23
	2	32.6 ±	2.3	29.4 ±	1.7	43.2 ±	1.0	6.48	2.01	11.83	0.17
	3	69.3 ±	5.5	64.8 ±	5.1	95.2 ±	9.7	5.40	3.35	11.58	0.29
	4	19.9 ±	1.5	16.0 ±	1.2	27.9 ±	3.4	6.64	1.62	11.9	0.14
	5	47.1 ±	4.9	41.7 ±	3.4	80.2 ±	7.5	5.86	2.68	11.7	0.23
	6	75.5 ±	7.4	69.8 ±	5.7	119.9 ±	7.1	5.07	3.73	11.51	0.32
	7	32.6 ±	2.3	29.4 ±	1.7	43.2 ±	1.0	6.48	2.01	11.83	0.17
	8	69.3 ±	5.5	64.8 ±	5.1	95.2 ±	9.7	5.40	3.35	11.58	0.29
	9	47.1 ±	4.9	41.7 ±	3.4	80.2 ±	7.5	5.86	2.68	11.7	0.23
RF + SM	**1**	51.7 ±	4.5	44.8 ±	4.5	64.4 ±	5.3	7.26	0.88	11.45	0.08
	2	32.3 ±	2.3	27.0 ±	3.4	47.9 ±	2.4	7.27	0.95	11.88	0.08
	3	72.5 ±	6.9	62.7 ±	6.7	96.1 ±	2.9	7.84	0.80	11.21	0.07
	4	24.3 ±	3.1	19.8 ±	1.9	44.3 ±	2.6	7.30	0.98	11.76	0.08
	5	51.7 ±	4.5	44.8 ±	4.5	64.4 ±	5.3	7.26	0.88	11.45	0.08
	6	85.2 ±	9.4	75.0 ±	6.2	116.5 ±	13.5	7.23	0.75	11.05	0.07
	7	32.3 ±	2.3	27.0 ±	3.4	47.9 ±	2.4	7.27	0.95	11.68	0.08
	8	72.5 ±	6.9	62.7 ±	6.7	96.1 ±	2.9	7.84	0.80	11.21	0.07
	9	51.7 ±	4.5	44.8 ±	4.5	64.4 ±	5.3	7.26	0.88	11.45	0.08
OP + SM	**1**	53.0 ±	4.0	43.9 ±	2.6	53.7 ±	1.5	7.13	0.91	10.82	0.08
	2	28.1 ±	7.5	26.5 ±	4.8	113.8 ±	25.1	7.01	0.84	10.27	0.08
	3	65.4 ±	5.8	61.4 ±	2.9	106.6 ±	46.6	6.85	0.74	9.41	0.08
	4	21.1 ±	2.1	19.4 ±	1.6	46.4 ±	0.6	7.38	1.01	11.64	0.09
	5	53.0 ±	4.0	43.9 ±	2.6	53.7 ±	1.5	7.13	0.91	10.82	0.08
	6	77.6 ±	5.9	73.5 ±	5.1	102.1 ±	8.8	7.12	0.80	9.95	0.08
	7	28.2 ±	7.5	26.5 ±	4.8	113.8 ±	25.1	7.01	0.84	10.27	0.08
	8	65.2 ±	5.8	61.4 ±	2.9	106.6 ±	46.6	6.85	0.74	9.41	0.08
	9	53.0 ±	4.0	43.9 ±	2.6	53.7 ±	1.5	7.13	0.91	10.82	0.08
FV + SM	**1**	58.1 ±	4.1	52.3 ±	3.2	97.6 ±	6.2	7.48	2.15	7.32	0.29
	2	41.5 ±	2.4	36.5 ±	1.5	74.5 ±	4.8	7.60	1.91	8.42	0.23
	3	73.5 ±	5.6	66.5 ±	5.8	111.9 ±	12.6	7.23	2.41	6.85	0.35
	4	30.8 ±	1.6	26.1 ±	1.4	63.2 ±	5.1	7.71	1.76	9.21	0.19
	5	58.1 ±	4.1	52.3 ±	3.2	97.6 ±	6.2	7.48	2.15	7.32	0.29
	6	83.5 ±	7.7	76.4 ±	5.2	148.7 ±	21.2	7.04	2.73	5.66	0.48
	7	41.5 ±	2.4	36.5 ±	1.5	74.5 ±	4.8	7.60	1.91	8.42	0.23
	8	73.5 ±	5.6	66.5 ±	5.8	111.9 ±	12.6	7.23	2.41	6.85	0.35
	9	58.1 ±	4.1	52.3 ±	3.2	97.6 ±	6.2	7.48	2.15	7.32	0.29

The chemical characterization of the considered substrates is reported in Table 3, for what concerns TS, VS, COD, pH, VFA and TA, in all EC. MS and FV showed higher VFA concentrations compared to RF and OP. In the case of MS, this probably was the cause of a slightly more acidic initial conditions, as compared to the other substrates. FV showed also lower TA and thereby higher VFA/TA ratios (Table 3.3). Generally, slight differences in pH were proportional to the dilution of the biomass with SM, depending on the EC.

More interestingly, Table 4 reports the characterization of the treated materials. Generally, a slight reduction of COD was observed, as expected, after fermentation process in all EC. pH diminished in the range 4.5 – 5.3 for those EC that showed high H_2 productivities (Table 4). When pH in the digester was higher than 5.3, H_2 productivity dropped below 20 $NL_{H2}\ kg^{-1}_{VS\text{-}added}$ (Table 3.4). In parallel, VFA/TA ratio in the range 1 – 2.2 corresponded to high H_2 yields, while for low productive EC, VFA/TA ratio was always found in the range 0.3 – 1.3 (Table 4). Contrarily, VFA concentration alone was not clearly related to productive EC. In many low-productive EC, high VFA concentrations were measured (up to 12 $g_{acetate}\ kg^{-1}_{ww}$, Table 4), as well as in some highly yielding EC, VFAs were found below 6 $g_{acetate}\ kg^{-1}_{ww}$ (Table 4).

Table 3.4 - Characterization of the fermented organic mixtures (after 1st stage treatment)

Sample	EC	TS *			COD			pH out	VFA	Alk	VFA/Alk
		g kg^{-1}_{ww}			g kg^{-1}_{ww}				g l^{-1}_{ww}	g l^{-1}_{ww}	
MS + SM	**1**	41.9	±	1.0	57.4	±	5.2	5.06	6.08	5.06	1.20
	2	38.8	±	1.1	41.9	±	5.4	5.23	5.12	5.54	0.92
	3	83.0	±	3.7	81.7	±	23.7	5.03	5.70	4.79	1.19
	4	13.9	±	2.1	38.5	±	6.0	5.57	6.61	5.97	1.11
	5	47.4	±	0.8	76.4	±	4.1	4.82	7.71	6.24	1.24
	6	77.1	±	4.2	109.5	±	6.7	4.77	7.55	6.88	1.10
	7	62.8	±	1.3	47.9	±	6.3	4.71	8.54	7.67	1.11
	8	83.7	±	2.1	104.6	±	1.3	5.10	10.28	9.19	1.12
	9	52.9	±	1.4	71.9	±	4.2	4.82	8.44	7.47	1.13
RF + SM	**1**	46.1	±	11.0	56.2	±	10.8	5.11	7.23	4.68	1.54
	2	31.8	±	5.4	32.2	±	5.1	6.16	4.30	6.7	0.64
	3	70.4	±	18.3	66.1	±	7.3	4.79	6.98	3.99	1.75
	4	18.1	±	1.1	35.2	±	6.7	6.46	4.81	4.91	0.98
	5	47.4	±	2.4	57.1	±	4.7	4.83	7.60	6.64	1.14
	6	81.9	±	1.1	114.6	±	9.5	4.76	12.24	11.37	1.08
	7	29.3	±	5.4	38.1	±	5.3	5.57	8.96	8.1	1.11
	8	68.1	±	0.3	59.5	±	2.0	4.78	10.05	8.75	1.15
	9	47.4	±	2.4	43.3	±	0.3	4.92	7.00	6.73	1.04
OP + SM	**1**	49.3	±	3.7	40.8	±	2.4	6.99	2.39	4.09	0.58
	2	26.1	±	6.9	24.6	±	4.5	6.35	3.89	4.72	0.82
	3	60.8	±	5.4	57.1	±	2.7	6.68	4.11	6.73	0.61
	4	19.6	±	2.0	18.1	±	1.5	7.72	1.40	3.98	0.35
	5	49.3	±	3.7	40.8	±	2.4	7.27	3.25	5.00	0.65
	6	72.1	±	5.5	68.4	±	4.8	6.07	4.19	3.80	1.10
	7	26.2	±	6.9	24.6	±	4.5	6.72	3.62	6.67	0.54
	8	60.6	±	5.4	57.1	±	2.7	7.18	2.84	3.85	0.74
	9	49.3	±	3.7	40.8	±	2.4	7.42	2.22	4.50	0.49
FV + SM	**1**	52.0	±	2.7	59.8	±	3.5	5.07	8.54	6.98	1.22
	2	29.2	±	0.6	44.6	±	7.2	5.63	10.50	9.14	1.15
	3	66.6	±	3.1	81.7	±	6.6	4.43	8.36	7.12	1.17
	4	28.4	±	1.5	43.4	±	3.7	5.64	11.23	8.29	1.35
	5	50.8	±	5.4	66.3	±	7.9	4.88	12.26	5.93	2.07
	6	56.4	±	2.1	113.9	±	38.6	4.57	11.27	6.54	1.72
	7	29.2	±	3.1	61.7	±	9.0	5.40	11.46	8.44	1.36
	8	57.2	±	1.9	96.3	±	20.9	4.34	11.87	7.29	1.63
	9	43.9	±	3.3	75.5	±	6.1	4.74	12.93	8.21	1.57

* OMC was not measured as VS, because consistent part of the VS of fermented materials are evaporated during drying

3.3.3 Methanogenic process yields

Optimized methane production was measured in batch reactors for raw (single-stage) and treated materials (2nd stage), excluding those EC that produced less H_2 than 30% of the best performing EC, in terms of H_2-yield (NL_{H2} $kg^{-1}_{VS\text{-}added}$). OP was completely excluded from the test, as soon as no H_2 was produced in any EC.

Methane yields resulted in the range 380 – 500 NL_{CH4} $kg^{-1}_{VS\text{-}added}$ for MS, 240 – 320 NL_{CH4} $kg^{-1}_{VS\text{-}added}$ for RF and 280 – 370 NL_{CH4} $kg^{-1}_{VS\text{-}added}$ for FV (Table 2). Average methane concentrations in the biogas were always relatively high for both second-stage and single-stage processes (Table 2). FV showed lower CH_4 contents, as compared to the other two substrates (55 – 59% v/v), while for MS and RF, methane concentrations ranges were 57 – 71% and 65 – 77% v/v, respectively (Table 2). Negligible differences in CH_4 concentrations were observed between second-stage and single-stage methanogenic production, for the same biomass (Table 2).

3.3.4 Energy recovery

Hydrogen and methane produced by the two-stage AD process (only the selected EC) were compared to the methane produced by the single-stage process, in terms of total energy recovered (Table 5).

Table 3.5 - Energy recovery (ER) per $kg_{VS\text{-}added}$ to the two-stage and one-stage AD systems

Mixture	EC	Two-stage ER						One-Stage ER	Increase of ER in two-stage % (MJ /MJ)
		1st stage (H_2)		2nd stage (CH_4)		Total two-stage		CH_4	
		MJ $kg^{-1}_{VS\text{-}added}$	% of total Two-stage	MJ $kg^{-1}_{VS\text{-}added}$	% of total Two-stage	MJ $kg^{-1}_{VS\text{-}added}$		MJ $kg^{-1}_{VS\text{-}added}$	
MS + SM	**1**	1.12 ± 0.10	7.7%	13.35 ± 0.88	92.3%	14.5 ± 1.0		15.17 ± 0.16	-4.6% b**
	2	1.54 ± 0.07	12.6%	10.67 ± 0.62	87.4%	12.2 ± 0.7		14.17 ± 1.32	-13.8% b
	3	0.54 ± 0.14	4.5%	11.61 ± 3.40	95.5%	12.2 ± 3.5		13.34 ± 0.84	-8.9% b
	4	0.16 ± 0.04	-*	-*	-*	-*		-*	-*
	5	0.74 ± 0.10	4.9%	14.28 ± 3.36	95.1%	15.0 ± 3.5		15.17 ± 0.16	-1.0% b
	6	0.28 ± 0.09	-	-		-		-	-
	7	1.27 ± 0.21	6.7%	17.71 ± 0.37	93.3%	19.0 ± 0.6		14.17 ± 1.32	34.0% a
	8	0.59 ± 0.03	3.9%	14.56 ± 0.13	96.1%	15.1 ± 0.2		13.36 ± 0.84	13.3% a
	9	1.25 ± 0.17	7.6%	15.19 ± 0.53	92.4%	16.4 ± 0.7		15.17 ± 0.16	8.4% a
RF + SM	**1**	1.63 ± 0.16	15.5%	8.88 ± 0.62	84.5%	10.5 ± 0.8		10.38 ± 0.55	1.3% b
	2	0.26 ± 0.05	-	-		-		-	-
	3	1.61 ± 0.19	15.7%	8.65 ± 0.48	84.3%	10.3 ± 0.7		9.84 ± 0.40	4.2% b
	4	0.08 ± 0.05	-	-		-		-	-
	5	1.48 ± 0.06	12.1%	10.74 ± 1.19	87.9%	12.2 ± 1.3		10.38 ± 0.55	17.8% a
	6	0.67 ± 0.49	5.7%	11.12 ± 0.10	94.3%	11.8 ± 0.6		9.52 ± 0.53	23.9% a
	7	0.07 ± 0.02	-	-		-		-	-
	8	1.23 ± 0.14	12.7%	8.44 ± 0.34	87.3%	9.7 ± 0.5		9.84 ± 0.40	-1.7% b
	9	1.40 ± 0.10	14.2%	8.45 ± 0.73	85.8%	9.9 ± 0.8		10.38 ± 0.55	-5.1% b
PO + SM	**1**	0.01 ± 0.01	-	-		-		-	-
	2	0.17 ± 0.03	-	-		-		-	-
	3	0.01 ± 0.01	-	-		-		-	-
	4	0.02 ± 0.01	-	-		-		-	-
	5	0.01 ± 0.01	-	-		-		-	-
	6	0.01 ± 0.01	-	-		-		-	-
	7	0.03 ± 0.02	-	-		-		-	-
	8	0.01 ± 0.01	-	-		-		-	-
	9	0.01 ± 0.01	-	-		-		-	-
FV + SM	**1**	0.34 ± 0.11	-	-		-		-	-
	2	0.01 ± 0.00	-	-		-		-	-
	3	0.42 ± 0.22	-	-		-		-	-
	4	0.08 ± 0.02	-	-		-		-	-
	5	1.58 ± 0.28	10.7%	13.12 ± 0.39	89.3%	14.7 ± 0.7		10.30 ± 1.14	42.7% a
	6	0.49 ± 0.18	3.9%	12.13 ± 1.14	96.1%	12.6 ± 1.3		10.27 ± 0.21	22.9% a
	7	0.20 ± 0.22	-	-		-		-	-
	8	1.26 ± 0.07	9.3%	12.22 ± 0.65	90.7%	13.5 ± 0.7		9.78 ± 0.48	37.8% a
	9	1.50 ± 0.34	11.6%	11.52 ± 0.97	88.4%	13.0 ± 1.3		9.88 ± 1.09	31.8% a

* Methane production was not considered for EC where H_2 yields resulted lower than 30% of the maximum H_2 yield

** letter a indicates significant (ANOVA for n=3. p<0.05) increase/decrease in ER for the two-stage AD, while letter b indicates non-significant differences

H_2 productions in the 1st-stage ranged from 0.5 to 1.6 MJ $kg^{-1}_{VS\text{-}added}$, corresponding to 4 – 16% of the whole two-stage AD system production (Table 5). The 2nd stage produced energy in the ranges 10 – 18, 8 – 11 and 11 – 14 MJ $kg^{-1}_{VS\text{-}added}$ for MS, RF and FV, respectively. The one-stage process generally produced a similar amount of energy, as compared to the 2nd stage. For this reason, when both 1st and 2nd stage were considered as sum (H_2 + CH_4) for the total ER of the two-stage system, in the majority of the cases, the two-stage resulted more productive than the one-stage (Table 3.5).

ANOVA was performed on two-stage ER *vs* one-stage ER, to look for the significant differences (p < 0.05, n = 3). The only case that showed significantly lower ER in the two-stage system was MS in EC2, while for all other EC, ER was never significantly higher in the one-stage (Table 3.5). On the other hand, nine EC showed significantly higher ER in the two-stage system, with the highest increase of 42.7% for FV in EC5 (Table 3.5).

3.4 Discussion

Relatively high bio-hydrogen yields were reached through the optimized first-stage approach. MS produced up to 120 $Sdm^3\ kg^{-1}_{VS\text{-}added}$, similarly to that found by previous authors [15, 16]. The same authors [16], reported also biohydrogen yields for various kinds of food waste in the range 60 – 130 $Sdm^3\ kg^{-1}_{VS\text{-}added}$ and the results of the present work were also coherent with previous experience by Tenca et al., obtained with the same substrate (FV + SM) [11]. The low productivity of OP was not a surprise. Other authors have worked on the same substrate and found similar biohydrogen yields (< 10 $Sdm^3\ kg^{-1}_{VS\text{-}added}$) [17, 18].

The obtained data give a robust contribution to demonstrate the general supremacy of the two-stage AD system, as compared to the one-stage approach. The method chosen in this work, i.e. to

optimize both hydrogenesis and methanogenesis, allowed overcoming inhibition/inefficiencies that could hide a general result, as happened in previous experiences (Schievano et al., 2012). In that paper, two- and one-stage gave the same ER, even if a clear partial inhibition of the two-stage was observed [10]. In this work, with the same substrate (FV + SM) used by Schievano et al. [10], the two-stage was demonstrated to be potentially (i.e. when both processes are optimized) more productive than the one-stage.

Generally, higher ER in the two-stage system didn't correspond to higher H_2 yields and, in any case, relatively high increases in ER (15 – 30%) were found even if H_2 counted for only 5 – 10% of the total ER. This confirms the hypothesis drawn by Luo et al. [6], according to which the real advantage created by the two-stage approach should be linked to more efficient methanogenesis, helped by pre-hydrolysis and pre-fermentation optimized in the first stage. Additionally, ER as hydrogen helps in increasing this advantage. In fact, to our knowledge, in a traditional one-stage process, comparable amounts of hydrogen are produced, while simultaneously converted into methane by hydrogenotrophic communities by the following reaction ($12H_2 + 4CO_2 \rightarrow CH_4 + 8H_2O$). This reaction, as all microbial process, require a fraction of energy for microbial methabolism and, as all microbial process, could sometimes and somehow be inefficient, depending on many factors. In the two-stage, instead, all energy contained in H_2 is recovered, thanks to physical separation of the fuel from the liquid phase.

This could be confirmed only by performing deeper characterization of the organic matter before and after the first-stage treatment, such as soluble carbon on total carbon, biodegradability tests and metabolite speciation.

3.5 Conclusions

This study was a first attempt, aimed at creating a new methodology for more comprehensively demonstrate the potentialities of the two-stage AD system. In future, this approach should be completed by deeper analytical procedures regarding both chemical and microbiological aspects.

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4. THREE-STAGE TECHNOLOGY TO COUPLE ANAEROBIC DIGESTION AND MICROBIAL FUEL CELLS

To be submitted

Abstract

Two-stage AD (anaerobic digestion) is a promising technology to improve the already well-established singl-stage process. To analyze the feisability of an amplification of the advantages coming from two-stage AD, a MFC (microbial fuel cell) system was paired with CSTR AD reactors, both single- and two-stage.

The use of an already tested biomass in a more realistic lab reactor allowed to prove that, for the considered conditions, no relevant differences are noticeable between the two technologies. Outputs coming from these reactors were then used as feeding for MFCs.

The overall energetic yield proved to be indeed higher in two-stage AD output fed MFC if compared to single-stage fed: MFC technology allows to increase the total volumetric energy production of 8,77% for two-stage and 4.39% for single-stage AD output fed MFCs.

Keywords: Two-stage anaerobic digestion, biohydrogen, biomethane, biomass, MFC

4.1 Introduction

Now more than ever energetic supplying and environmental impact represent a concern for world society. Energy consumption practically doubled in the last three decades with 80% of energy obtained from fossil fuels (IEA, 2005).

This leads to search solutions to some prioritarial problems, such as the exhaustion of limited, non renewable fossil sources and the consequences of environmental pollution and climate change. In this context the struggle for efficient, sustainable and renewable sources is becoming a major concern.

While single stage anaerobic digestion (AD) already proved to be a reliable way to obtain energy in form of methane gas from bacterial degradation of biomass, the two-stage process is showing

interesting perspectives as a viable technology to coproduce hydrogen and methane in two separate, in-series reactors from a vaste variety of waste materials (Ting and Lee, 2007; Xie et al., 2008). According to some authors, splitting the reaction at the stages of hydrolysis/acidogenesis and methanogenesis could enhance the overall reaction rate, maximize biogas yields, and make the process easier to control, both in meso- and thermophilic conditions (Blonskaja et al., 2003; Liu et al., 2006).

Hydrogen is emerging as a promising fuel, thanks to its high specific heat and virtually null dangerous emissions (Nath and Das, 2004). Despite present technological limitations and challenges, hydrogen is considered to be a possible energy carrier for the future, and developing sustainable methods to obtain hydrogen from renewable sources, other than fossil-fuel based technologies, is necessary in order to fully achieve the potential economical and environmental benefits. The chance to obtain this efficient fuel from a renewable, eco-sustainable process (compared to the other fossil fuel-based pathways, i.e. steam reforming, thermal cracking, coal gasification and partial oxydation) enhances its potential in the biological fuel landscape.

While it is vastely known, from previous studies, how operative parameters (temperature, pH etc.) and possible pretreatments can influence the biomethane production, work is still to be done on the analysis of how chemical composition of biomass affects hydrogen production.

According to Lay et al. (2003), carbohydrates-rich biomass show a hydrogen yield up to 20 times higher than proteins- and fat-rich biomass. For the latter, an optimum pH point for hydrogenic fermentationt is 6, while the former have an optimum at 5.

More recent studies (Kim et al., 2012) focused on improving operative conditions in order to obtain a good hydrogen production rate from fat substrates. Anaerobic digestion was carried out in reactors fed with lard (with obvious high lipidic content); these reactors were equipped with a stirring and a CO_2-removal system. Hydrogen production was noticed in both situations; still, the conditions of production of the system provided with CO_2 remover was about 3 times higher than with the stirrer by itself; also a better consumption of VFA (Volatile Fatty Acids) was noticed. Combining both

methods allowed an even higher hydrogen production and no methane in gas. These data proved how even a lipidic-rich biomass could lead to a significative hydrogen production under specific operative parameters. Gallert and Winter (1997) analyzed how termophilic bacteria, such as those implied in two-stage AD hydrogenic phase, can stand higher levels of ammonia from protein degradation compared to mesophilic ones, thus suggesting a possibile suitability of protein-riche biomass for hydrogen production.

Another possible source of clean, ready-to-use energy are bioelectrochemical systems (BESs), especially of the microbiological kind (MBESs), such as microbial fuel cells (MFC) and microbial electrolysis cells (MEC). They in fact allow the combined production of electric energy (MFC), biohydrogen (MEC) and the depuration of wastewaters throug organic load reduction(Rabaey *et al*., 2010). An additional advantage from this technologies is the lower greenhouse gases emission from energy production, compared to traditional systems (Rabaey & Verstraete, 2005). At present, MFC are the most widespread BESs technology (Franks & Nevin, 2010): energy in organic substrates' chemical bounds is turned to electricity by anaerobic bacteria (*Geobacter, Shewanella, Proteobacter, Pseudomonas*) (Du *et al*., 2007). Organic matter oxydation paired with terminal electron acceptor (TEA, usually oxygen) allows continuous electric production (Logan, 2008). Recently, Schievano et al. (2012) compared mono-stage and double-stage AD working on a semi-continuous system for hydrogen production (first stage) and in a CSTR reactor for the second stage and single stage AD. This study goes further in the analysis of two-stage anaerobic digestion and proposes a three-stage concept, using MFCs in series after AD and allowing a comparison of one-stage, two-stage and three stage anaerobic systems.

4.2 Materials and methods

4.2.1 Anaerobic Digestion (Single-, First- and Second stage)

Three continuous flow stirred tank reactors (CSTR), in ''wet'' AD conditions (total solids < 10% w/w), were used in this study and the reactor designs are reported in Figure S4.1. The

wet CSTR was chosen, as soon as it is one of the most used type of AD in full-scale applications. The two-stage process consisted of a 0.25 L hydrogen-producing reactor with 0.2 L working volume (R1) and a 3 L reactor with 2 L working volume for methane production (R2). The single-stage process consisted of a 3 L reactor with 2 L working volume (R3). The same feeding mixture was added both to R1 and R3 after the removal of an equal amount (measured as wet weight) of effluent from the reactors. Hydraulic retention time (HRT) were of 3, 30 and 33 days for the reactor R1, R2 and R3, respectively (Figure 4.1).

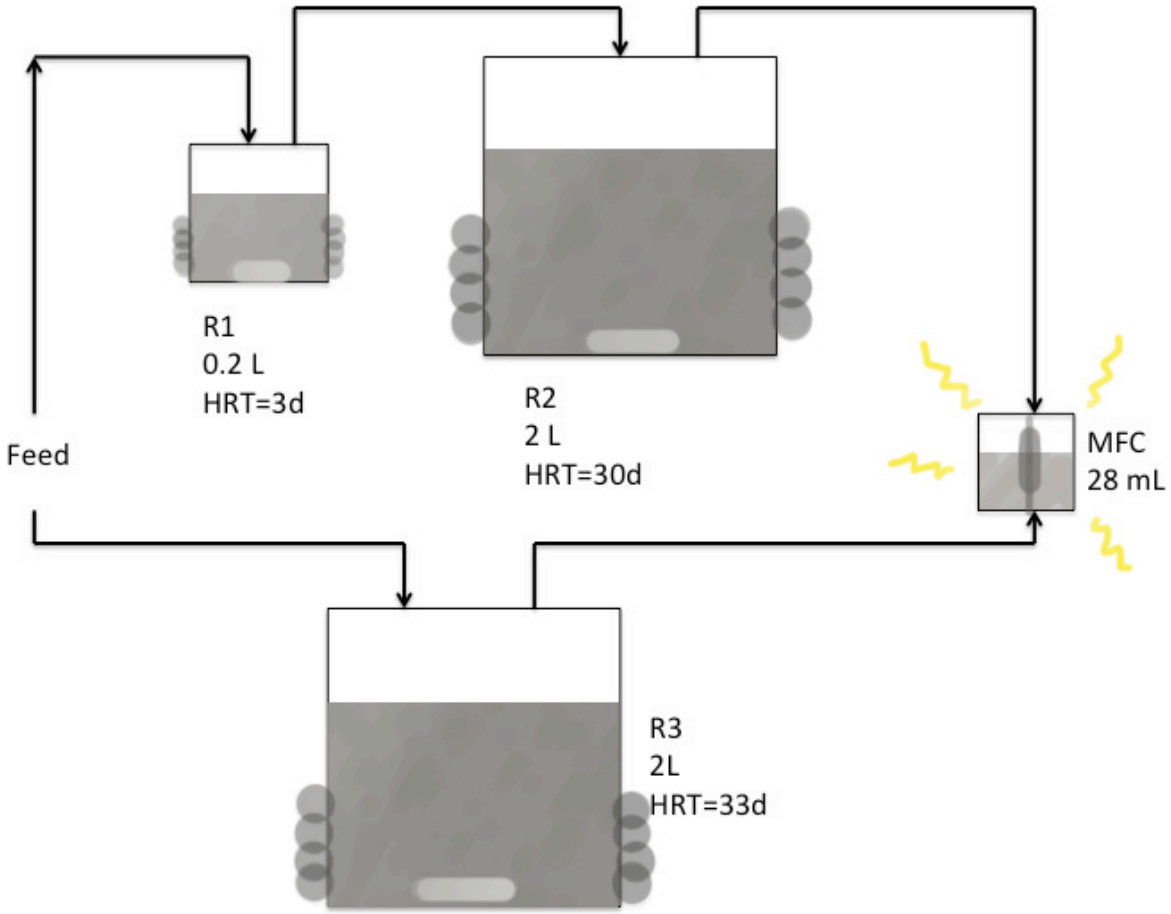

Fig.4.1: AD reactors and MFC structure.

The feeding procedure was in semi-continuous regime, i.e. twice a day the digestate was removed and equal volume of feeding mixture was inserted in each reactor. The operational HRT of R1 was chosen according to previous experiences on optimized bio-hydrogen production from organic waste materials (Schievano et al., 2013). HRTs of the methanogenic

phases were chosen based on the batch biochemical methane potential tests performed on the same organic mixture by Schievano et al. (2013). The overall HRT of two- and single-stage processes were equal (33 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 (over 24 h) cumulated amount was accounted. Biogas composition, also, was determined daily, using a gas chromatograph: H_2, CH_4 and CO_2 relative concentrations (v/v) were measured. Methane and hydrogen productions were calculated as daily cumulated production volume.

4.2.2 Third stage: Electrodes and bioreactors

Single-chamber, air–cathode MFCs containing graphite fiber brush anodes were constructed as previously described (Logan et al., 2007). Each reactor consisted of a liquid chamber 4 cm long by 5 cm in diameter, with a liquid volume of 28 mL. Brush anodes were made of a core of two titanium wires with graphite fibers (PANEX33 160 K, ZOLTEK) cut to 2.5 cm in outer diameter and 2.5 cm long. Each brush had an estimated surface area of 0.22 m or 18, 200 m^2 m^3-brush volume for the brush, with 95% porosity (Logan et al., 2007). The cathodes (3.8 cm diameter, 7 cm^2 total exposed surface area) were made by applying a platinum catalyst (0.4 mg $Ptcm^2$, BASF) on the liquid-facing side of a 30 wt.% wet-proofed carbon cloth (type B-1B, BASF, US), while four PTFE diffusion layers were added on the air-facing side (Cheng et al., 2006). Cells were connected to an external resistor (Rex = 1 kΩ); the whole system was hence connected to a multimeter (2700;

Keithley, United States) to measure outcoming voltage. Data were stocked on a computer.

Digestate from single stage and from second reactor of two-stage AD were centrifuged; the liquid phase was diluted 1:2 in water and used as feeding for MFC reactors.

All tests were performed in duplicate.

4.2.3 Chemical analysis

Both biomass, feeding mixtures and digestates were characterized. pH and conductivity were measured using pH meter and conductivity meter (PC 2700, Eutech Instruments, Netherlands). Total Solids (TS) and Volatile Solids (VS) content was evaluated according to standard procedure (Sluiter et al., 2003). TKN (Total Kjeldhal Nitrogen) was calculated on fresh material, while N-NH_4+ content was used to evaluate the total protein content of samples (Bremner, 1996).

Total chemical oxygen demand (TCOD), biological oxygen demand (BOD_5) were measured according to Standard Methods (APHA, AWWA, WPCF, 1998).

Polarization curves were obtained by varying the external resistance (10–10,000 Ω) every 30 min. and measuring the cell voltage (Sciarria et al., 2013)

4.3 Results and discussion

4.3.1 AD reactors

In table 4.1 chemical characterizations of rice middlings and swine manure are shown.

Tab.4.1: biomass characterization

	pH	**TS** g kg $^{-1}$	**VS** g kg TS $^{-1}$	**Tot Alk** mg $CaCO_3$ l^{-1}	**TVFA** mg CH_3COOH l^{-1}	**NH_4^+** mg l^{-1}	**TN** g kg $^{-1}$
Rice middling	--	890±3	879±1	--	--	--	--
Swine manure	6.93±0.06	21.2±0.3	733±1	7752±153	5285±112	1290±21	1.67±0.08

Rice middlings confirmed to be a particularly dry biomass, with a total solids content of 890 g kg^{-1}, while for swine manure it was way lower, 2.1 g kg^{-1}; rice middlings volatile solids content was 879 g $kg^{-1}$of dry matter compared to 733 g kg^{-1} of swine manure. For the latter pH, VFA, alkalinity and

nitrogen content were measured too.

In figure 4.2 results from biogas and chemical analysis of single stage reactor are shown.

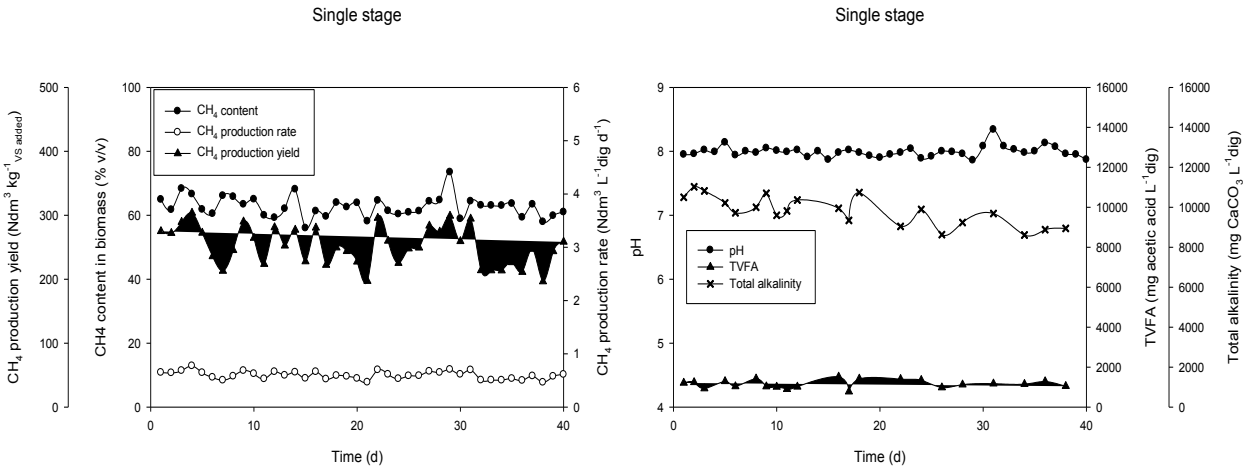

Fig. 4.2: Methane content, rate and yield (left) and pH, VFA and alkalinity (right) for single stage reactor.

CH_4 production rate was mainly stable during the whole test, with a value around 17.4 Ndm^3 L^{-1} dig d^{-1}. CH4 production yield has an average value of 251 Ndm^3 kg^{-1} VS added and reached its maximum (305 Ndm^3 kg^{-1} VS added) on day 4, while CH_4 content in biomass peaked on day 30 (73.5 %v/v). pH was overall stable (pH=8), except for a peak (8.4) on day 31; VFA content was low (average 1156 mg acetic acid L^{-1} dig) and alkalinity ranged from 8610 to 11023 mg $CaCO_3$ L^{-1} dig. Figures 4.3 and 4.4 show the trends of two-stage AD reactor, first- and second stage respectively.

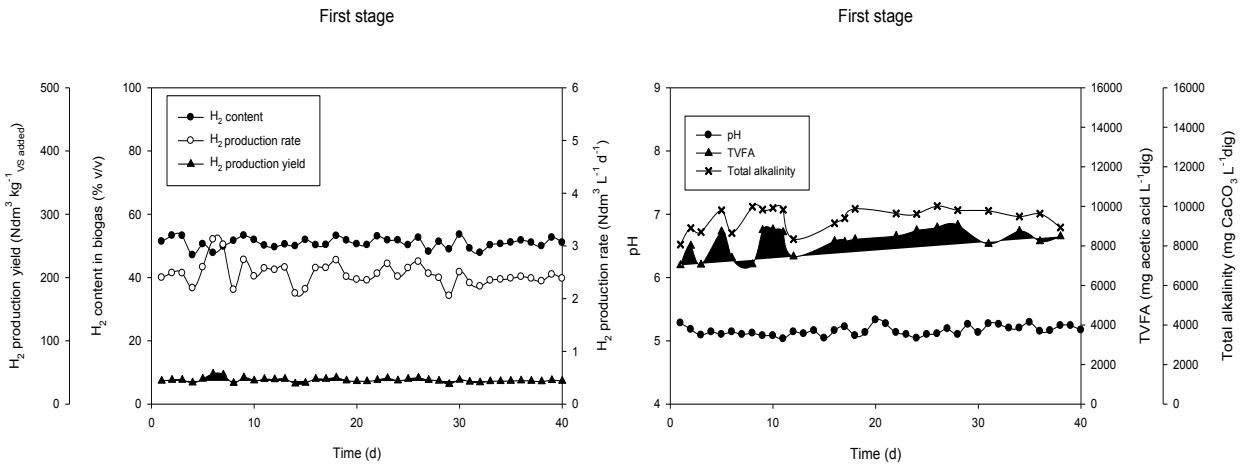

Fig. 4.3: Hydrogen content, rate and yield (left) and pH, VFA and alkalinity (right) for first stage reactor.

For hydrogenic reactor, methane production was nearly null (due to process characteristics) and thus was not reported. Still, biogas produced proved to be rich in hydrogen, with an average H_2 content of 50.9%, with a peak of 53.6%, and a production rate ranging from 2.05 $Ndm^3\ L^{-1}\ d^{-1}$ to 3.12 $Ndm^3\ L^{-1}\ d^{-1}$. Still, H_2 production yield was stable and low (0.38 $Ndm^3\ kg^{-1}$ VS added). pH value was lower than single stage (as expected) and hovered around 5. VFA and alkalinity patterns were similar in trend, with average values of 8221 mg acetic acid L^{-1} dig and 9397 mg $CaCO_3\ L^{-1}$ dig respectively.

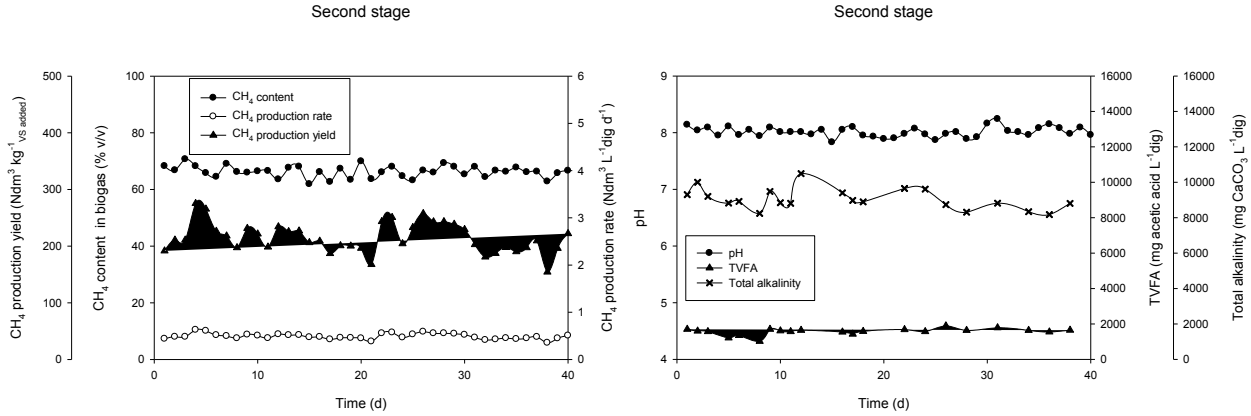

Fig 4.4: Methane content, rate and yield (left) and pH, VFA and alkalinity (right) for second stage reactor.

In figure 4.4, gas production (on the left) and pH, VFA and alkalinity values (right) are shown. CH_4 content in biogas reached an average of 66,7 %, with a maximum of 70,7%. Average produtcion rate was 14.3 $Ndm^3\ L^{-1}$ dig d^{-1}, ranging from 10.3 $Ndm^3\ L^{-1}$ dig d^{-1} to 18.4 $Ndm^3\ L^{-1}$ dig d^{-1}. As for CH_4 production yield, a peak of 270 $Ndm^3\ kg^{-1}$ VS added, with an average of 214 $Ndm^3\ kg^{-1}$ VS added.

pH value was stable (8) while average VFA and alkalinity content were 1756 mg acetic acid L^{-1} dig and 9117 mg $CaCO_3\ L^{-1}$ dig respectively.

Tab.4.2: chemical analysis on feeding mixtures and digestates

			Two-stage		Single-stage
		Feeding	First (F)	Second (S)	
TS	$g\ kg^{-1}$	114.3±0.5	107.7±7.8	53±9.0	59.8±6.3
VS	$g\ kg^{-1}{}_{TS}$	857.4±2.05	846.9±9.0	764.6±21.9	769.7±0.6
TS reduction	% of fed		5.8±1.1	50.8±6.8	47.7±5.5
				53.6±5.2 (F+S)	
VS reduction	% of fed		5.6±0.9	55.6±7.9	53.03±6.1
				58.6±8.3 (F+S)	
VS-OLR	$g\ VS\ L^{-1}\ d^{-1}$		65.3±5.8	2.28±0.08	2.36±0.3
				2.36±0.2（F+S）	
pH		7.09±0.08	5.16±0.08	8.01±0.09	7.99±0.09
TVFA	$mg_{CH3COOH}\ kg^{-1}$	4825±96	8221.4±656.5	1755.9±680.9	1156.1±199
TA	$mg_{CaCO3}\ kg^{-1}$	7103±155.5	9397.6±579	9116.9±827	9397.6±734
TVFA/TA	$mg_{CH3COOH}\ mg^{-1}{}_{CaCO3}$	0.7±0.02	0.88±0.05	0.19±0.06	0.12±0.02
$N\text{-}NH_4^+$	$g\ kg^{-1}$	1.25±0.034	1.19±0.1	1.82±0.1	2.05±0.2
TN	$g\ kg^{-1}$	2.98±0.05	2.9±0.3	2.5±0.1	2.8±0.1
$N\text{-}NH_4^+$/TN	%	43±2	41±3	74±4	73±2

The two-stage process shows a better total solids reduction when considering both reactors when compared to the single-stage one. A similar decrease trend is witnessed for volatile solids; this data are significant to testify that two-stage AD allows a better biomass degradation. Still, the VFA content in the output from second stage reactor is significantly higher than that from single stage one, thus pointing out a chance for a better overall degradation, reachable through different, longer retention times.Total nitrogen and ammonia contents are, on the other hand, similar.

Table 4.3 shows bigas and energy production and comparison between single- and two-stage

processes.

Tab.4.3: biogas (volume, composition, rate and yield) production and energetic yield from single- and two-stage reactors.

		Two-stage				Single-stage	
		F		A		B	
		Average (40 d)	Var. (per d)	Average (40 d)	Var. (per d)	Average (40 d)	Var. (per d)
Volumetric biogas production rate	$Ndm^3 L^{-1}_{dig} d^{-1}$	4.84	0.2	0.74	0.01	0.95	0.01
Hydrogen content in biogas	% v/v	50.9	2.57	＜0.1		＜0.1	
Methane content in biogas	% v/v	＜0.1		66.7	9.3	62.7	10.7
Carbon dioxide content in biogas	% v/v	49.1	2.4	33.1	10.7	37.1	11.6
Volumetric hydrogen production rate	$Ndm^3_{H2} L^{-1}_{dig} d^{-1}$	2.5	0.05	Udl		Udl	
Volumetric methane production rate	$Ndm^3_{CH4} L^{-1}_{dig} d^{-1}$	Udl		0.49	0.005	0.59	0.005
Hydrogen/methane production yield	$Ndm^3_{H2/CH4} kg^{-1}_{VS added}$	37.7	10.9	214.5	21	251.4	25
Volumetric energy production rate	$kJ L^{-1}_{dig} d^{-1}$	15.65	1.87	14.5	4.8	17.4	4.8
Energetic yield	$MJ kg^{-1}_{VS added}$	0.38	0.001	6.37 6.75 (F+A)	0.92 0.7	7.39	0.86

These data don't allow to state that a significant difference in energy production between the two processes exists. The difference (energetic yield seems higher in single stage than in two-stage process) may not depend on actual process issues but on the unsufficient number of collected data. When calculating energy obtained from biogas from the two processes in a cogenerator, both result in a value of 8 W.

4.3.2 MFC

Input and output from MFC underwent a chemical characterization to analyze organic matter depletion.

Tab.4.4: feeding and output characterization for single stage process.

		Average in	Average out	Δ	Δ (%)
COD	g l^{-1}	6.80±1.5	5,60±0.4	1.2	17.5
BOD	mg l^{-1}	6,70±1.2	5±1.3	1.6	24.5
NH3	mg l^{-1}	772±52	593±50.7	179	23.2
NO3-	mg l^{-1}	25.3±2.5	26±6.3	-0.7	-2.9
TKN	%	991±31.8	774±72.5	217	21.9
Total Alkalinity	mg_{CaCO3} kg^{-1}	3658±348	3295±59.5	363	9.9
Inorganic Alkalinity	mg_{CaCO3} kg^{-1}	2584±221	2396±207.3	188	7.3
VFA	$mg_{CH3COOH}$ kg^{-}	572±7.78	394±73.9	178	31.1
pH		8.10±0.1	8.23±0.22	-0.1	-1.6
Conductivity	mS	7.88±0.78	7.16±0.36	0.7	9.2

Data show a diminuition in most of parameters. COD goes from a value of 6.80 g l-1 in feeding to 5.6 g l^{-1} in output, with a Δ of 1.2 g l^{-1} (17.5 %). A similar trend is witnessed for most parameters, with a Δ of 24.5 % for BOD, 23.2 % for NH_3 and 21.9% for TKN . Both total and inorganic alkalinity decrease of 9.9% and 7.3% respectively. VFAs value goes from 572 to 394 mg CH_3COOH l^{-1}, with a depletion of 31.1%. Conductivity shows a diminuition of 9.2%. Opposite trends witnessed for NO_3^- and pH (Δ=-2.9% and Δ=-1.6%) may depend not on process but on collected data.

Tab.4.5: feeding and output characterization for second-stage process.

		Average in	Average out	Δ	Δ (%)
COD	$g\ l^{-1}$	6.90±0.2	5,40±0.3	1,6	22.4
BOD	$mg\ l^{-1}$	6,53±0.23	4.8±0.1	1.8	27
NH3	$mg\ l^{-1}$	771±0.2	629±36.1	142	18.4
NO3-	$mg\ l^{-1}$	24.10±3.3	21.8±2.4	2.4	9.8
TKN	%	912±41.7	739±18.2	173	19
Total Alkalinity	$mg_{CaCO3}\ kg^{-1}$	3644±410.2	3216±173	428	11.7
Inorganic Alkalinity	$mg_{CaCO3}\ kg^{-1}$	2233±12.4	2335±324.8	-101.6	-4.5
VFA	$mg_{CH3COOH}\ kg^{-}$	803±96	397±80	406	50.5
pH		8.33±0.02	8.23±0.1	0.1	1.1
Conductivity	mS	7.72±0.21	7.09±0.5	0.5	8.2

Trend for process running on second-stage output show some differences when compared to single-stage one. Most parameters decrease during run in MFCs, except for inorganic alkalinity (Δ=-4.5%). NH_3 decrease (18.4%) is lower than that of single-stage process, as well as TKN (19%) and Conductivity (8.2%). Still, it is interesting to point out that parameters more closely connected to process' efficiency (such as COD, BOD and VFA) present an higher Δ (22.4%, 27% and 50.5% respectively) when compared to the other process.

A better degradation of organic matter, as seen in higher COD and BOD Δ from second stage feeding, is correlated to a better energy production; this evidence shows a general improved performance from two-stage process.

For every test four voltage cycles were performed. Both experiments allowed to reach similar voltage peaks (≈ 0.50 V) (data not shown), but different power peaks, as shown in figures 4.5 and 4.6.

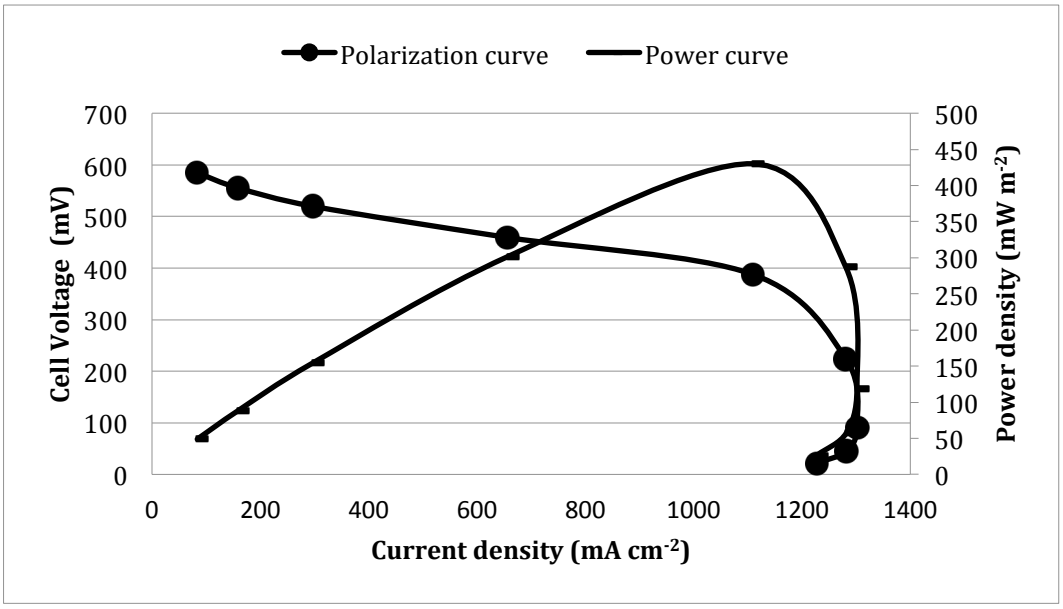

Fig.4.5: Polarization and power curves from single-stage output fed MFC

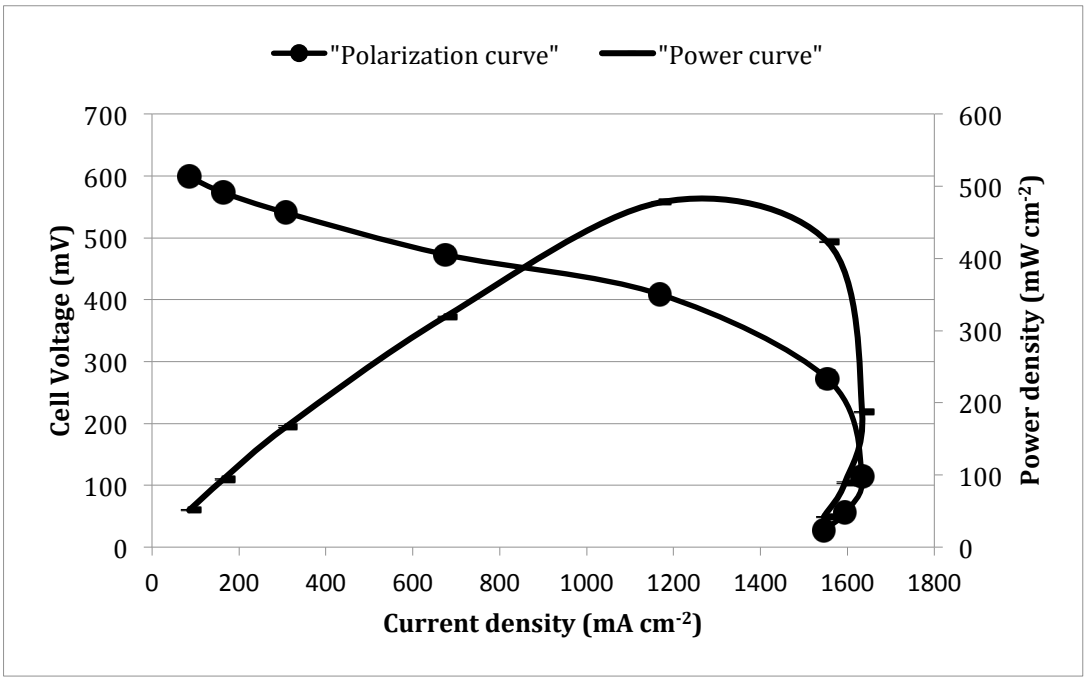

Fig.4.6: Polarization and power curves from single-stage output fed MFC.

As shown in the graphs, second-stage fed MFC resulted in a better performance, with a value of

power density of 477 mW/m^2 (500 Ω), while single-stage one only 430 mW/m^2 (500 Ω). These values refer to the surface as m^2 of the catode used for the essay and are representative of the cell-system functioning. What is more interesting is to relate energy production to our reactors' volume, in order to compare the total energy production from both technologies (AD and MFC). Volumetric power of our MFC system was determined as described previously by Logan (2006 e 2008), the volumetric power calculated from polarization curves is 12.2 W/m^3 (430 mW/m^2, 500 Ω) for single stage and 13.3 W/m3 (477.37 mW/m^2,500 Ω), for second stage fed MFC. To confront the energy production from the two systems (AD reactors and MFC), volumetric power was evaluated for AD reactors too (based on volumetric gas production and calorific power), finding values of 182 W/m^3 for single stage reactor and 171 W/m^3 for two-stage reactor, considering the energy loss due to gas immission in a cogeneration engine. MFC technology therefore allows an improvement in power generation of 4.39% for single stage, while for two-stage the increase is of 8.77%. From this point of view, a three-stage systems shows better performances when run on output from second-stage AD.

4.5 Conclusions

Single- and two-stage AD don't show significant differences in energy production if considered singularly. Still, a better energy production from two-stage AD (deriving from a more performant organic matter degradation) is visible when digestates coming from AD reactors are used as feeding mixtures for MFC technologies.

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5. ANAEROBIC DIGESTION FOR THE PRODUCTION OF HYDROGEN AND METHANE: FROM LABORATORY TO FULL SCALE

5.1 Introduction

Each year in Italy 220 million cubic meters of biogas are produced, against a potential of 54 billion cubic meters; produced biogas is currently used primarily by co-generators for the production of electricity and heat *in situ*.

The term “biomethane" refers to a biogas that underwent a refining process to reach a methane concentration of 95%; it is used as a biofuel for motor vehicles as much as fossil methane gas.

Biogas is produced by biological breakdown of organic matter without oxygen in a process known as anaerobic digestion (AD).

AD can take place in a controlled environment (digester) with a methane content in biogas produced equal to 55-65%, or even in landfills due to the decomposition of waste: in this case the biogas or landfill gas contains a percentage of methane up to 45%.

The main raw materials used in AD process are:

-Urban wastewaters and sludge;

-Slaughterhouse and farming wastewaters;

-Wastes from markets and food industry;

-Domestic organic wastes;

-Gardening wastes;

Specific crops such as forage or switchgrasstoo can be conveniently used for the process of AD.

However, the most common raw material in Europe is sewer wastewater, used for the integrate depuration and AD process.

In Great Britain, about 75% of sewage is treated this way, and the resulting gas is used to produce heat and electricity. In Lille, France, the city's sewer system is a source of raw material to produce biogas, which is then refined to be used as fuel for buses.

Waste and by-products from agriculture, food industry and dedicate crops can be used on-site in small digesters, as happens in Germany or Italy.

Raw biogas can be combusted directly to produce heat or electricity after having undergone minimal processing of filtration and purification. The biogas can be used in the same vehicles that employ commonly natural gas or methane of fossil origin.

Anaerobic digestion is a well established process that has been spreading in recent decades as one of the best, most versatile and easily reproducible technologies for the production of renewable energy from biomass, especially from waste biomass of the various sectors of human activity. Currently, the production of biogas is associated with the production of electrical energy, through the direct utilization of the biogas in internal combustion engines (on-site). These solutions in the vast majority of cases do not allow to fully utilize the thermal energy co-generated, which amounts to about 50% of the total energy in the bio-fuel.

In the future, as already happening in Germany, Sweden, Austria and Switzerland, biomethane will be distributed as biofuel in the national pipe network and used in cogeneration to obtain 100% of its energy content and / or as biofuel for vehicles. Biomethane, in fact, is one of the bio-fuels with less impact on air quality (very low pollutant emissions), even when compared with other biofuels such as bioethanol and biodiesel.

Hydrogen

The hydrogen sector has long been at the center of important initiatives , thanks to the characteristics of high sustainability of this energy carrier . Already many promising utilizations for this gas are reknown.

Hydromethan

The chemical procedures known as hydrogen fuel injection (HFI) are a series of processes that include various technologies , such as the use of mixtures of molecular hydrogen / methane (hydromethane), or molecular hydrogen / octane to improve combustion in internal combustion

engines , especially gasoline , through the use of hydrogen (added to the fuel or generated by the engine through an alternator and a rectifier. Hydromethane is a gas mixture, which currently can be composed of up to 30 % hydrogen and 70% methane, which has also been proposed as fuel in internal combustion engines for various means of transport . This mixture has the advantage of a more rapid ignition and a more complete combustion . It allows to reduce by 50% the emissions of nitrogen oxide and CO.

In the early Seventies the Jet Propulsion Laboratory of NASA published a series of articles on the benefits of the addition of hydrogen in the combustion of hydrocarbons in conventional internal combustion engines . The NASA HFI system generates hydrogen during running and adds it to the gasoline (composed mainly of but also benzene , cyclohexane, n-decane , toluene) creating a mixture of fuels with a behavior comparable to a better octane . A better combustion is obtained, which leads to increased power, lower fuel consumption and less pollution . It also increases the durability of the engine as it reduces carbon deposits (anthracenes and fullerenes) in the cylinders due to the incomplete combustion.

Biohydrogen

However , the production of hydrogen is currently linked to traditional combustion of fossil fuels; in this case hydrogen can only be considered an energy carrier , and not a source .

Still, when its production takes place through renewable sources, hydrogen can actually be considered an energy source.

AD can be used as a biological technology for hydrogen production. The anaerobic fermentation is thus one of the most studied processes thanks to its high yield potential (Benemann , 1996) .

Biohydrogen production occurs in the acidogenic phase, during which the microorganisms metabolise simple sugars to produce volatile fatty acids (acetic acid and butyric acid) and, in the end, hydrogen. Stoichiometrically, each mole of glucose consumed is converted into 4 or 2 moles of hydrogen, depending on the metabolic product is acetic acid or butyric acid (Levin et al., 2004) .

At the end of hydrogenic phase, the substrate has a high content of volatile fatty acids and therefore

represents an ideal matrix to be used in a further, methanogenic fermentation. Hawkes et al. (2002), talking of two-stage anaerobic digestion, originally thought for the production of methane only, propose a modification to allocate the first reactor to the production of hydrogen and to use the effluent of this phase in a second reactor in cascade to produce methane.

The concept has more recently been tested by some research groups with results of great interest. Kraemer and Bagley (2005) tested the process in a two-stage lab scale reactor using an artificial glucose-based substrate and obtaining a yield of 180 Nm^3 and 320 Nm^3 for ton of volatile solids respectively., Liu et al. (2006) carried out a study with more interesting substrates (diluted urban wastes). In this case average yields were of about 50 Nm^3 ton^{-1}VS of hydrogen and 500 Nm^3 ton^{-1} VS of methane, with unitary gas production respectively of 0.07 Nm^3h and 0.11 Nm^3 h^{-1} per m^3 of reaction.

In order to compare the yield of the two stage process with that of the traditional fermentation, the authors fed the same substrate to a single stage reactor operating in parallel to the double-stage. In this case, the average production of methane was 0.09 Nm^3 h^{-1} per $m^3$of reaction.

It is truly remarkable to note that, under comparable conditions, the two-stage process has produced over 25% more energy than the single-stage fermentation.

These results have raised interest in the testing of the two-stage fermentation also on pilot plants. The AIST (The Japan Institute of Advanced Industrial Science and Technology) has completed in 2005 a plant capable of treating about 60 kg per day of organic residues in food mixed paper. The average gas productions were 0.5-1 Nm^3 d^{-1} of hydrogen and 5-10 Nm^3 d^{-1} of natural gas (AIST, 2005). Further analysis showed an increase of energy production by approximately 20% compared to traditional methanogenic fermentation, in addition to a reduction in process time in the order of 40%. In conclusion, on the basis of current knowledge documented by the international scientific literature, it can be said that the two-stage anaerobic fermentation:

• is a reliable technique, although optimizable, for the production of hydrogen from renewable sources;

• is capable of producing significant quantities of bio-hydrogen from organic waste materials, potentially harmful to the environment;

• is energetically more efficient than the traditional anaerobic fermentation and the amount of energy generated can be further increased through the optimization of the process;

• promises to be the process of producing renewable hydrogen economically sustainable and suitable for micro-territorial distribution.

Despite the vast amount of dedicated studies, little is known on how AD could actually work on a real, full scale (not pilot) plant. Thanks to the cooperation with Cascina Castagna (S. Angelo Lodigiano, LO) it was possible to analyze the functioning of the first european full-scale plant from the very beginning of its activation. A lab-scale process was carried out in parallel to ensure an optimal feeding rate and composition.

5.2 MATERIALS AND METHODS

5.2.1 Lab reactors

To optimize the process and ensure stability from a biological point of view, a lab scale simulation was carried out. Two digesters (C and D) were fed with two different mixtures based on the biomass' availability in the full-scale plant.

Startup inoculum for semicontinuous tests was collected from a 10 l lab-scale reactor fed with glucose and kept working at termophilic conditions (55°C) for five months in order to ensure a stable hydrogen production.

Semicontinuous tests were carried out in pirex Wheaton batches with an operative volume of 250 ml (first stage) and 3 l flasks (second stage, single stage). Temperature was kept stable at 55°C with a syliconic tube sleeve with circulating water connected to an eated bath. Reactors operatad on a magnetic stirrer (stirring rate=60 rpm) with an hydraulic retention time of 3 days. Rice middlings was diluted with swine manure.

The volumetric gas production was measured with a tilting system connected to a counter; 500 ml

of gas caused one tilt, and the overall number of movements was reported on the counter.

Gas was collected from the reactors with a graduated syringe and analyzed with a micro gas-cromatograph (μGC; Model 3000A-μGC, AGILENT-SRA Instruments).

Both feeding mixtures are based on swine and bovine sludge from Cascina Castagna, with the addition of grain.

Mixture 1 provides an addition of corn silage, while mixture 2 of ryegrass silage.

Each of those mixtures has been used in two different conditions, based on different concentrations of the biomasses.

In table 1 wet weight biomass' amounts and digester conditions are shown.

Tab.5.1: C and D feeding chemical composition for both experimental phases (1 and 2).

		10/04—04/06		08/06—10/07	
		Lab scale digestors			
		C1	D1	C2	D2
Swine sludge	% (w/w)	45	48	46.8	44.3
Bovine sludge	% (w/w)	45	48	46.8	44.3
Grain	% (w/w)	1.85	1.85	3.8	2.8
Corn silage	% (w/w)	8.0	2	-	-
Ryegrass silage	% (w/w)	-	-	2.6	8.6
HRT	day	3	3	3	3
Organic load	g VS L^{-1} d^{-1}	24.9	19.6	25.4	25.9
Organic concentration	gVS L^{-1}	74.9	59.8	76.1	77.8
Alkaline load	g $CaCO_3$ L^{-1} d^{-1}	2.51	2.67	2.61	2.48
Alkalinity	g $CaCO_3$ L^{-1}	7.55	8.03	7.82	7.43
VS/Alk	g VS / $gCaCO_3$	9.93	7.44	9.72	10.47

All reactors were fed with a mixture of swine and bovine sludge, grain, corn silage (for first test) or ryegrass (second test). Hydraulic retention time was three days for both trials. Reactor C sported a higher organic load and concentration and volatile solids/alkalinity ratio in condition 1; its alkaline load and alkalinity, instead, were inferior than reactor D in first test. In condition 2 these parameters were inverted.

5.2.2 The plant

The plant consists of two separated reactors. Feeding mixture is conveyed in the first reactor where a partial hydrolisis takes place. The biogas produced –rich in hydrogen- is collected and the output used as feeding for a second reactor. Here the second stage of anaerobic digestion leads to the producion of methane-rich biogas.

This plants allows a cogeneration efficiency up to 90%; 90% of biomass used for alimentation come straight from Cascina Castagna farm.

Both biogases undergo a process of integrated upgrading to cleanse them from water, CO_2, H_2S and other useless compounds. Upgraded biomethane is later used for national web injection (5 bar), cogeneration and, with hydrogen addition, as a car fuel.

The fuel produced is used in a cogeneration station (see fig.), able to generate 250 kW of electric and 300 kW of thermical energy (with smoke recovery), clean and eco-friendly. A prototype of a second, smaller upgrading station is built in the plant to purify the produced gases and use them as car fuels (bioH_2, bioCH_4, hydromethane). A small van operating in the farm is already hydromethane-powered, but the same technology will apply, in the future, for agricultural vehicles.

The plant, operating since February 2013, underwent a process of startup (March-July). In this period, samplings were carried out on a weekly base for the characterization of biomass, while gas produced and digestates were sampled more often.

During April, May and June, 2013 the sperimental start-up phase of the biological process of hydrogen production through dark fermentation was carried out in the full-scale plant in Cascina

Castagna (LO).

The plant is composed as shown in the table 2. Pictures from the plant are shown in supporting informations.

Tab.5.2: plant's structure.

	Function	**Volume**
R1	First stage, acidogenic fermentation	150 m^3
R2	Second stage, methanogenesis	1800 m^3

From April to July 2013 the plant underwent a startup phase; during this phase feeding and working conditions were monitored on a daily basis, as shown in table 3.

Tab. 5.3: feeding mixture's composition and HRT for reactor 1.

		10/04—04/06	08/06—10/07
		Mixture 1	Mixture 2
Swine sludge	% (w/w)	47.1	46.8
Bovine sludge	% (w/w)	47.1	46.8
Grains	% (w/w)	3.7	3.7
Corn silage	% (w/w)	2.1	-
Ryegrass silage	% (w/w)	-	2.6
HRT (R1)	days	2.83	2.71
HRT (R2)	days	32	32
Organic load	g VS L^{-1} d^{-1}	26.5	31.91
Organic concentration	gVS L^{-1}	74.9	86.6
Alkaline load	g $CaCO_3$ L^{-1} d^{-1}	2.79	2.79
Alkalinity	g $CaCO_3$ L^{-1}	7.88	7.57
VS/Alk	g VS / g$CaCO_3$	9.51	11.44

Feeding parameters show values similar to those of lab scale reactors (C1 and D2), yet adjusted according to biomass' availability on field in time.

Hydraulic retention times varied when compared to lab reactors, due to different reactors' volumes.

In graphs N and N it is possible to see the exact timing feeding trend, both as wet weight and organic matter.

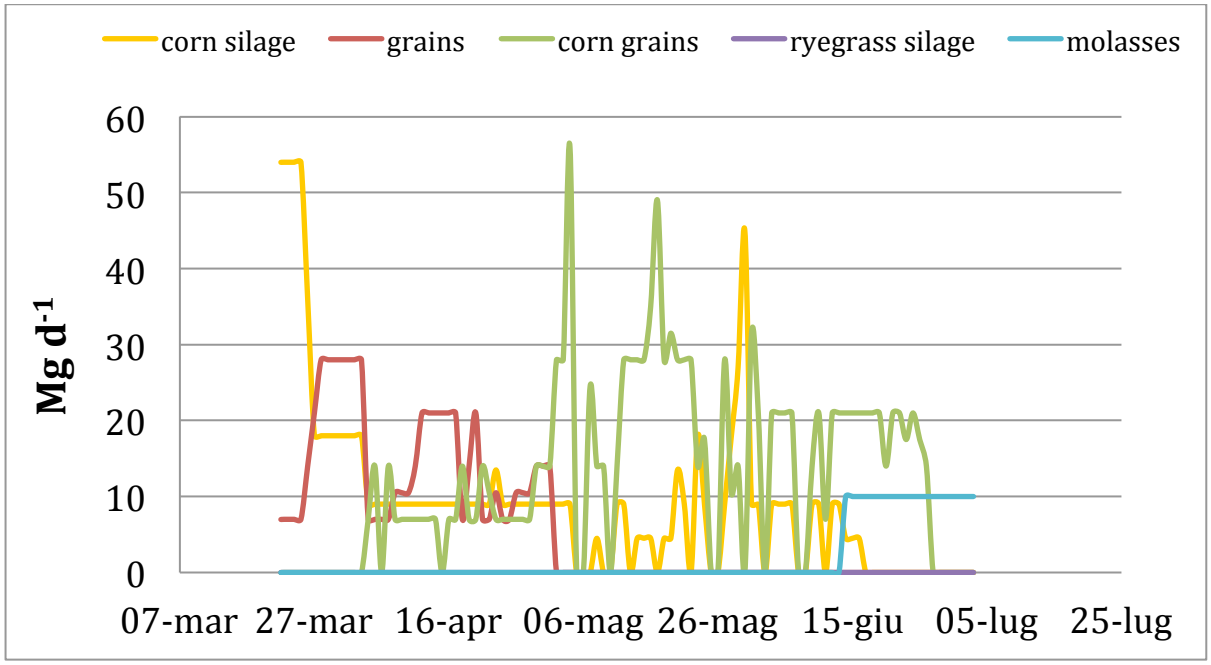

Fig.5.1: composition of feeding mixture in time.

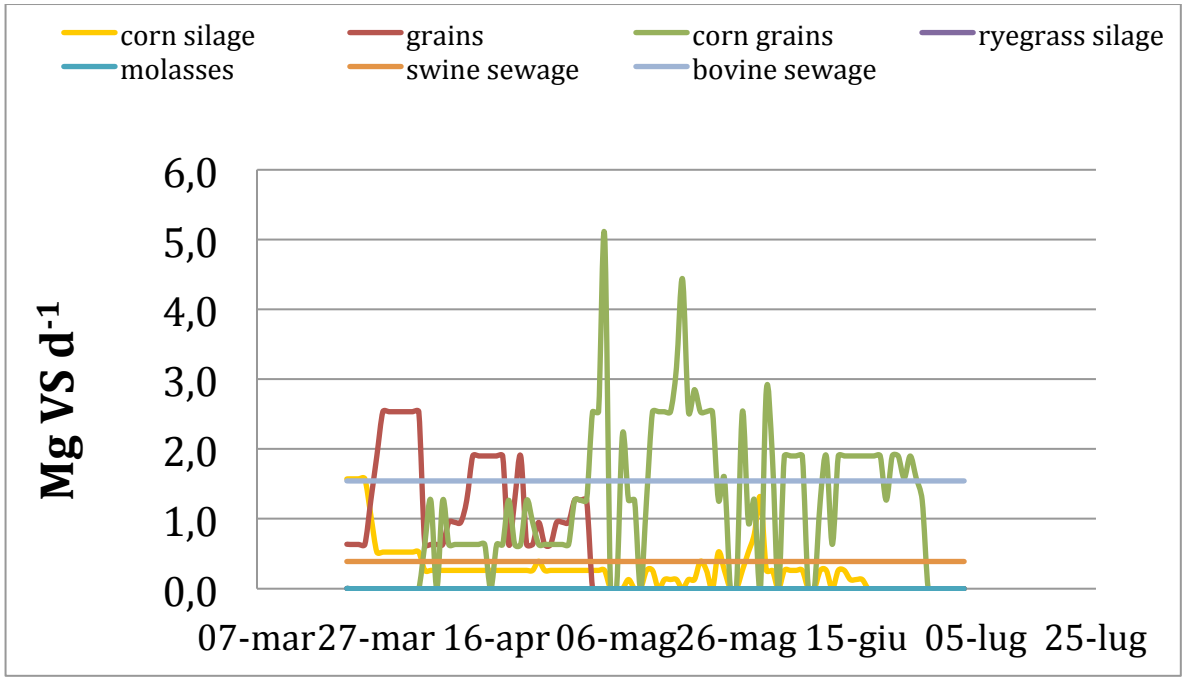

Fig.5.2: organic composition of feeding mixture in time.

Grains were only used for the first period, being later substituted by corn grain. The organic load from swine and bovime sludge was stable throughout the whole startup period.

5.2.3 Chemical analysis

Total Solids (TS) and Volatile Solids (VS) content was evaluated according to standard procedure (Sluiter et al., 2005). TKN (Total Kjeldhal Nitrogen) and N-NH_4 content were measured (Bremner, 1996).

Volatile fatty acids (VFA) content and alkalinity were evaluated according to Lahav et al. (2002).

5.3 RESULTS

Biogas productions, VFA concentrations and alkalinity for lab scale reactors are shown in the following graphs.

Mixture 1 shows an acceptable process stability only in digestor C.

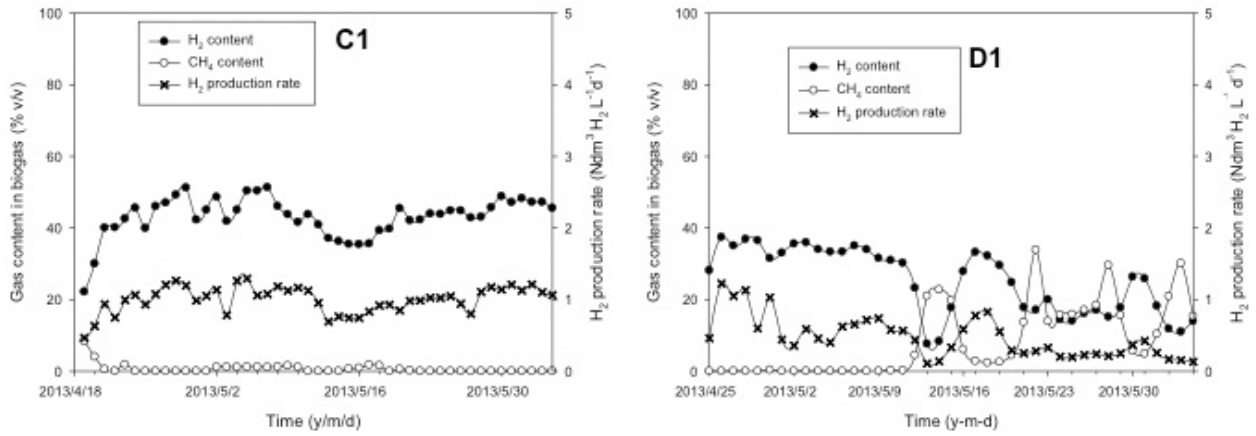

Fig. 5.3: Hydrogen and methane content in biogas from mixture 1 (lab scale)

During phase 1, reactor C showed an higher H_2 content and production rate (41% and 0.91 Ndm^3 $H_2L^{-1}d^{-1}$), with a methane production nearly null. On the other hand, reactor D not only resulted in lower H_2 productivity, with an average H_2 concentration of 19.1% and rate of 0.36 Ndm^3 $H_2L^{-1}d^{-1}$, but the biogas produced was 16.4%.

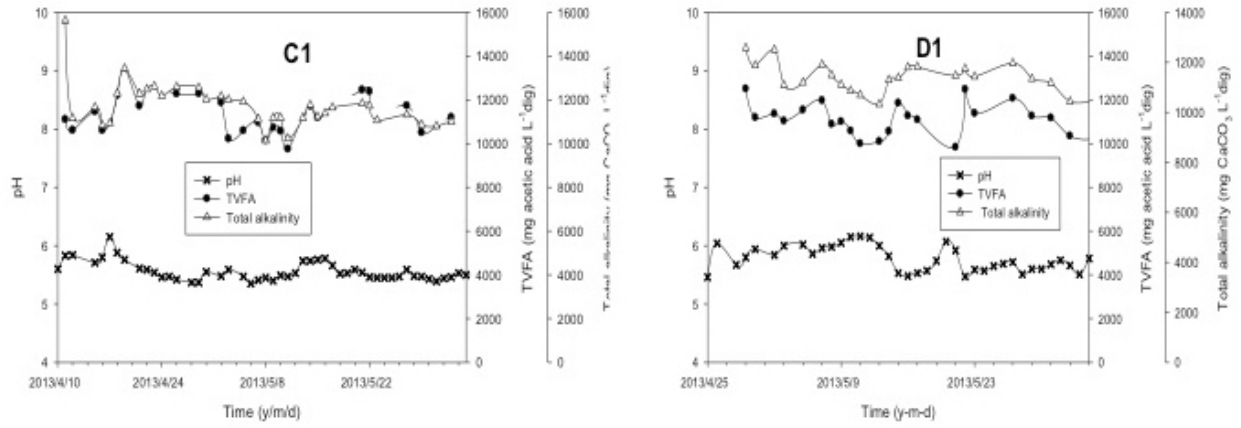

Fig. 5.4: pH, TVFA and alkalinity of output from phase 1 (lab scale)

In both reactors, pH, alkalinity and volatile fatty acids follow a similar trend. pH ranged from 5.5 to 6.2 for both C and D, while C's alkalinity and VFA are in general higher (average 11520 mg l^{-1} CH_3COOH and 11771 mg l^{-1} $CaCO_3$ for C, 10494 mg l^{-1} CH_3COOH and 11407 mg l^{-1} $CaCO_3$for D).

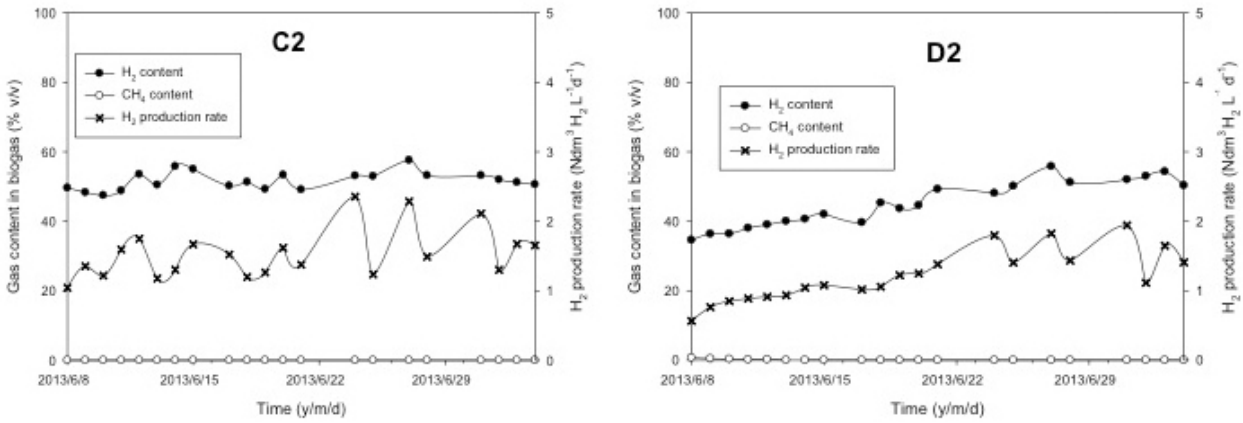

Fig. 5.5: Hydrogen and methane content in biogas from phase 2 (lab scale).

During phase 2, for both reactors CH_4 production was null. Reator D showed a more regular trend. In reactor C, average H_2 content in biogas was 49.8% and H_2 produtcion rate 1.3 Ndm^3 $H_2L^{-1}d^{-1}$. Reactor D, on the other hand, showed an H_2 content in biogas produced of only 30.5% and a production rate of 0.65 Ndm^3 $H_2L^{-1}d^{-1}$.

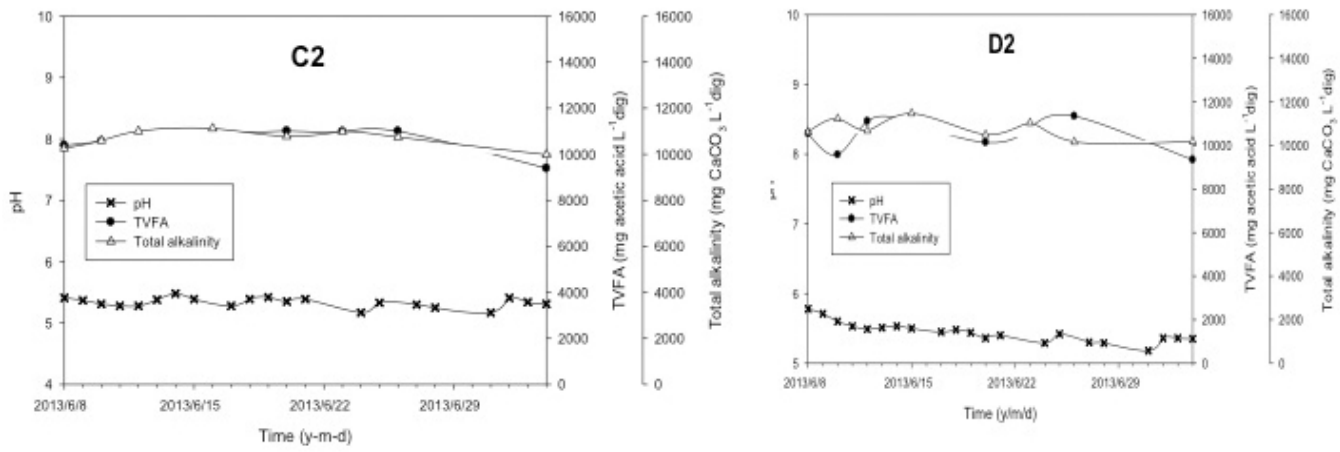

Fig.5.6: pH, TVFA and alkalinity of output from phase 2 (lab scale)

Average pH was 5.44 for reaactor C and 5.64 for reactor D. VFA concentration in reactor C was 10758 mg l^{-1} CH_3COOH and alkalinity 10781 mg l^{-1} $CaCO_3$, slightly higher than values from D (10506 mg $l^{-1}CH_3COOH$ and 10773 mg l^{-1} $CaCO_3$). Still, there is less difference between the two reactors when compared to condition 1.

In parallel, feeding and biogas from full scale plant were anlyzed.

In figures 7 and 8 trend in biogas quality and H_2/CH_4 and CO_2 concentrations in reactor R1 are shown. Quantitative measurements were only available since June.

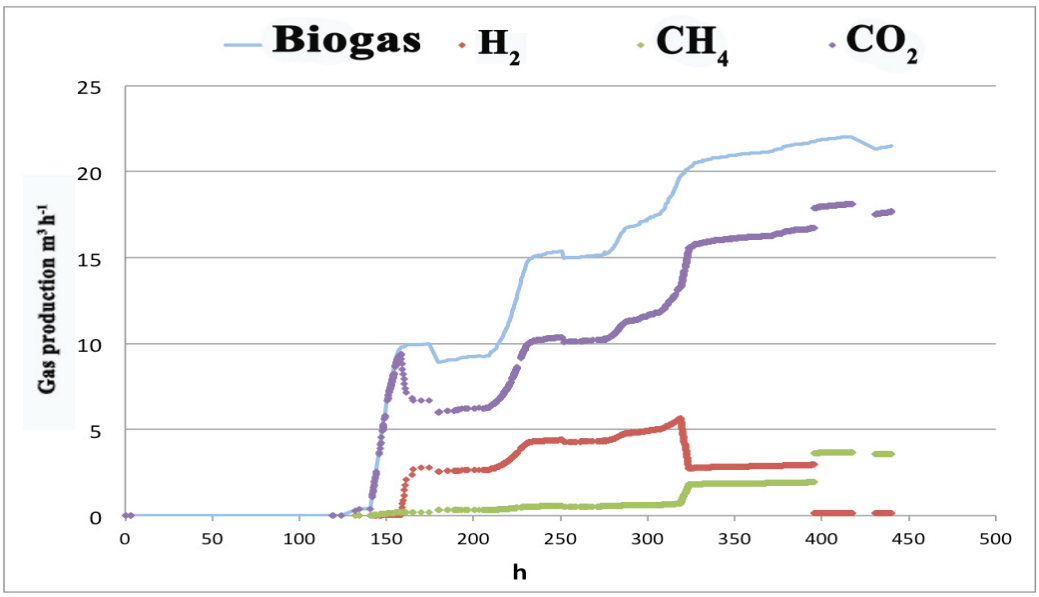

Fig 5.7: hourly total and specific biogas volumetric production.

As seen in picture above, biogas volumetric production both total and specific for different

compounds (H_2, CH_4 and CO_2) was measured on an hourly base through a blowing system. Daily, this biogas was sampled through a syringe and measured with a MicroGC; percentages of different gases are shown in figure N.

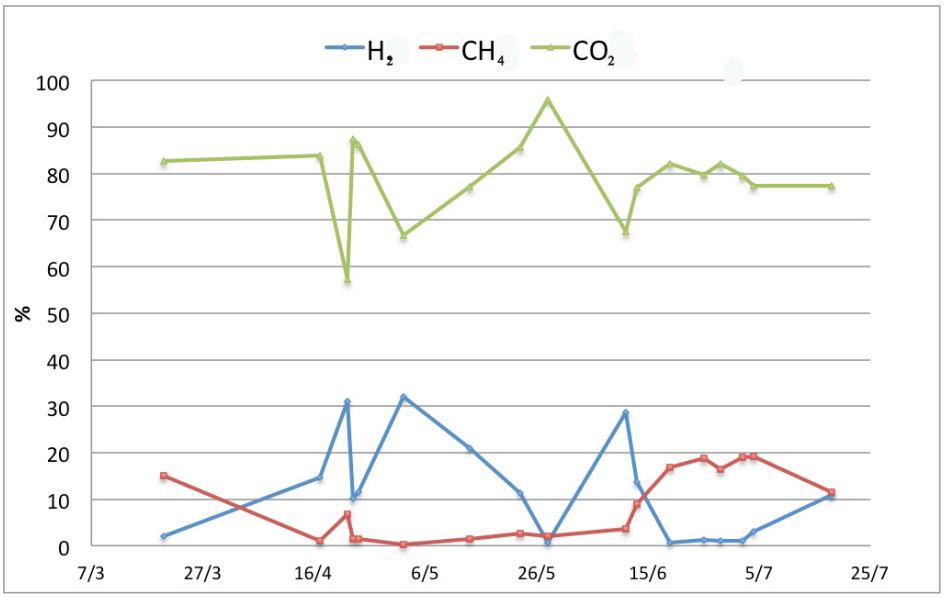

Fig. 5.8: percentages of H_2, CH_4 and CO_2 in biogas from R1

It is possible to see that hydrogen and methane productions follow an inverse trend; between 9 and 11 of june an increase in methane volume is witnessed, paired with an equal hydrogen diminuition. While the trend is not so clearly distributed when considering percentages, it is still evident how H_2 and CH_4 content follow a similar trend as volumes.

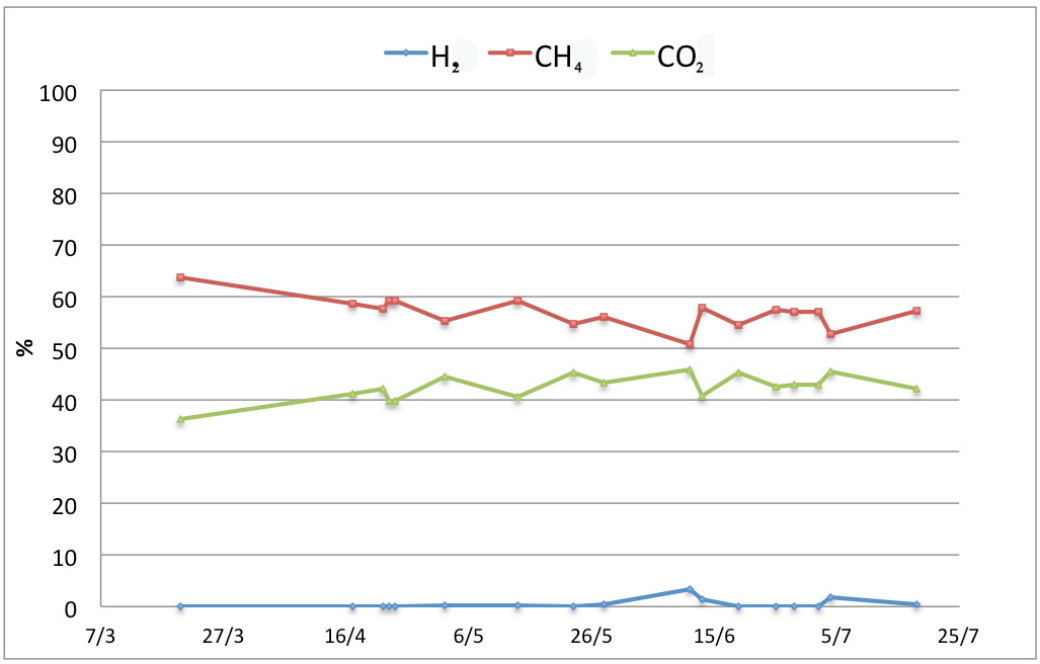

Fig.5.9: percentages of H_2, CH_4 and CO_2 in biogas from R2

R2 shows inverted trends for CH_4 and CO_2 percentages; CH_4 never went under a value of 50%, indicating overall good process conditions. H_2production was nearly null.

H_2S content is higher in R1 than in R2, as shown in figure 5.10; this may depend on a series of problems in the reactor structure (leaks and blocked pumps).

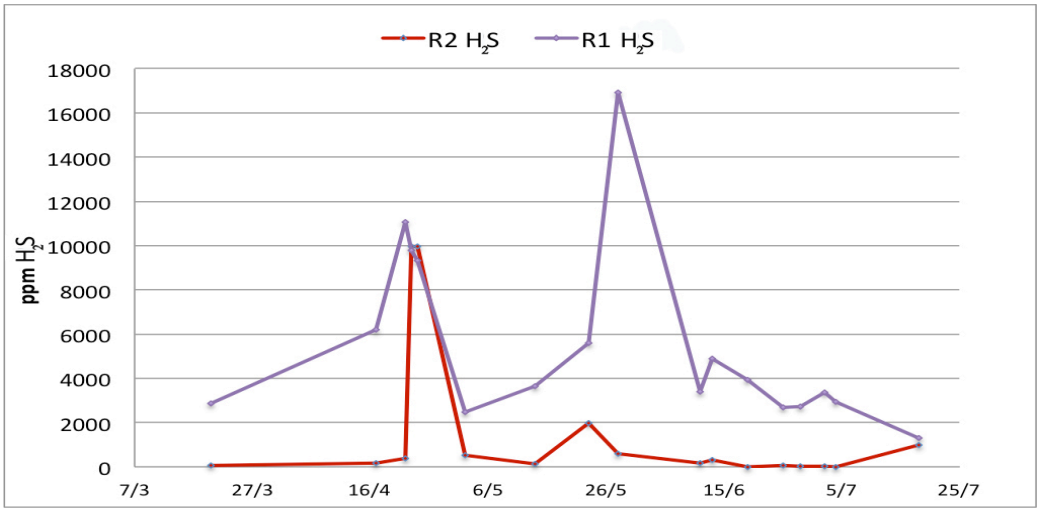

Fig.5.10: H_2S content in both reactors.

Chemical parameters in digestors were measured as shown in tab 5.4 (R1) and 5.5 (R2).

Tab. 5.4: chemical characterization of output from reactor R1

Date	*pH*	*VFA (mg/kg acetic acid)*	*Total alkalinity (mg/kg $CaCO_3$)*	*VFA/alk R1*	*NH_4^+ (mg/L)*	*TKN (g/kg)*	*TS g/kg*	*VS g/kg dry*
21-mar	6.83	6064.00	10206.00	0.59	2059	2.70	56.20	
17-apr	5.38	6896	7915	0.87			57.87	
22-apr	5.05	11575	12055	0.96	1718			
2-may	5.4	9891	10217	0.97			37.51	794.30
14- may	5.57	10206	10467	0.98				
28- may	4.62	11070	11683	0.95	1745	2.10	58.21	906.81
30- may	4.63	4166	4659	0.89	1528	2.48	35.40	829.79
4-jun	5.34	11825	12330	0.96	1804	2.80	49.25	842.21
7- jun	5	13445	13720	0.98	1805	2.78	85.70	876.39
11- jun	5.49	7237	7865	0.92	1398	2.63	41.53	838.24
13- jun	6.08	8786	9374	0.94	1452	2.65	41.95	837.65
19- jun	5.62	9301	10235	0.91	1461	2.62	36.85	742.46
25- jun	5.43	11761	11208	1.05	1543	0.28	42.73	
27- jun	5.41	11291	10991	1.03	1660	0.31	66.43	
2-jul	6.38	10109	9872	1.02	1697	0.29	67.47	
4- jul	6.45	9533	9966	0.96	1729	0.30		
18- jul	6.13	12624	11349	1.11	1875		76.14	85.48

Tab.5.5: chemical characterization of output from reactor R2.

Date	*pH*	*VFA (mg/kg acetic acid)*	*Total alkalinity (mg/kg $CaCO_3$)*	*VFA/alk R1*	*NH_4^+ (mg/L)*	*TKN (g/kg)*	*TS g/kg*	*VS g/kg dry*
21-mar	7.99	709	10043	0.07	2295	3.10	39.10	
17-apr	7.68	924	7473	0.12			37.12	
22-apr	7.66	565	9402	0.06	1731			
2-may	7.83	713	10179	0.07			32.86	760.25
14- may	7.59	1958	10524	0.19				
28- may	7.39	2052	10731	0.19	2220	2.83	35.17	813.81
30- may	7.55	2621	9932	0.26	2138	2.93	31.83	762.30
4-jun	7.56	2186	10978	0.20	2047	2.98	31.17	769.63
7- jun	8.14	1045	11214	0.09	2195	2.43	29.70	757.30
11- jun	7.6	1786	8879	0.20	2004	2.83	32.48	821.66
13- jun	7.69	1893	9502	0.20	1960	2.88	33.82	782.32
19- jun	7.48	2156	9521	0.23	2133	2.74	30.88	803.67
25- jun	7.64	2835	10672	0.27	1994	0.31	33.36	
27- jun	7.54	2731	11023	0.25	2043	0.31	37.31	
2-jul	7.71	2224	10645	0.21	2114	0.28	37.74	
18-jul	8.27	1074	11177	0.10	2450		34.68	75.35

pH values range more widely in R1 (from 4.62 to 6.45); in R2 this variation is less evident (7.48 to 8.27). This is reflected in VFA content, reaching a maximum of 13445 mg kg^{-1}acetic acid in R1 compared to R2 highest, 2731 mg kg^{-1} acetic acid. Alkalinity range is less dramatic (from 4659 mg kg^{-1} $CaCO_3$ to 13720 mg kg^{-1} $CaCO_3$in R1, from 7473 mg $kg^{-1}CaCO_3$to 11214 mg $kg^{-1}CaCO_3$in R2). For R1, NH_4^+ content goes from 1452 mg L^{-1} to 2059 mg/L and from 1731 mg L^{-1} to 2450 mg L^{-1} for R2; TKN ranges from 0.28 g kg^{-1} to 2.78-3.10 g kg^{-1} for both reactors.

Output from R1 shows a higher content of total solids (35.4-85.7 g kg^{-1}) compared to R2 (29.7-39.1 g kg^{-1}).

pH, alkialinity and VFAs trends for both reactors are shown in figures 5.11 and 5.12.

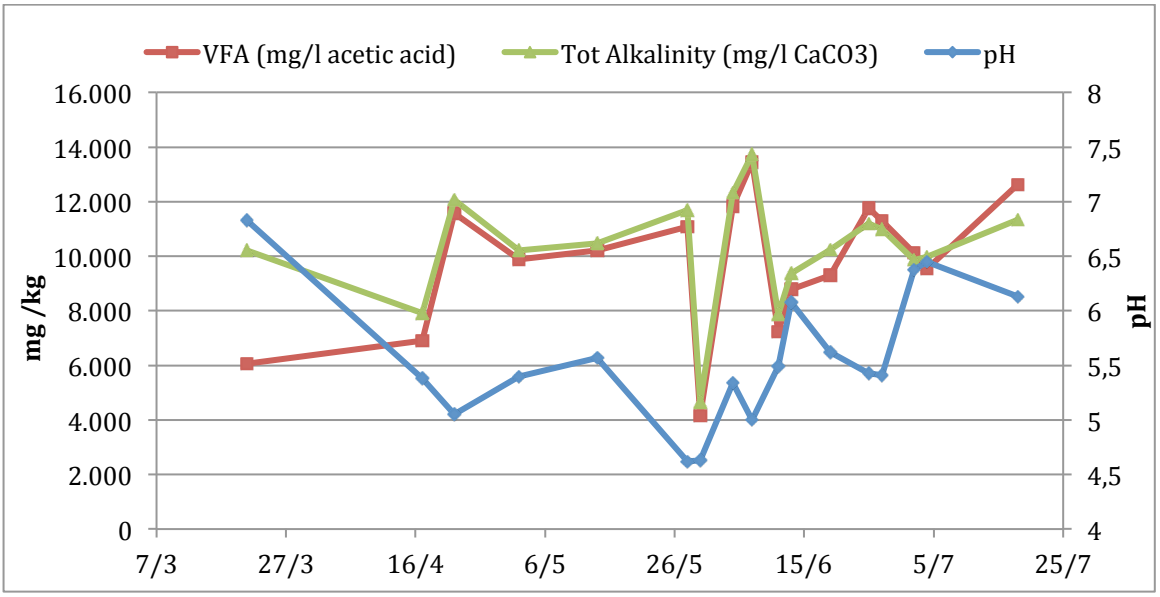

Fig.5.11: VFAs, Alkalinity and pH trend in R1

VFA and alkalinity follow a similar trend, reaching a maximum of 14000 mg/l CH3COOH and mg/l of CaCO3 on early June. Both find their lower point at the end of May (4000 mg/l CH3COOH and mg/l of CaCO3 respectively). pH is instead less stable, ranging from 4.6 to 6.9. This irregular trend reflects the difficulties met in plant's administration.

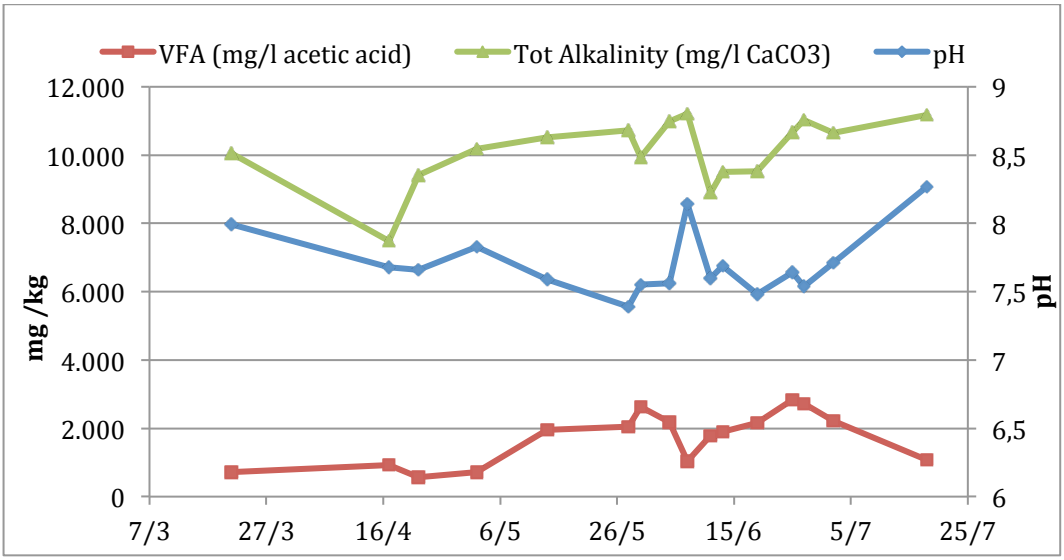

fig.5.12: VFAs, alkalinity and pH in R2.

Trends are in general more regular in R2, following the overall more linear and already tested process of AD for methane production only. VFA content is –as expected- lower than in R1, with a peak of 2900 mg/l CH_3COOH; alkalinity ranges from 7700 to 11800 mg/l $CaCO_3$, still higher than R1. This is consistent with general higher pH and VFA concentration in hydrogenic reactor.

In fig. 5.13 trends for VFA/alkalinity ratio is shown; R1 shows higher values due to general higher VFA content. Values seem to follow an opposite trend for the two reactors.

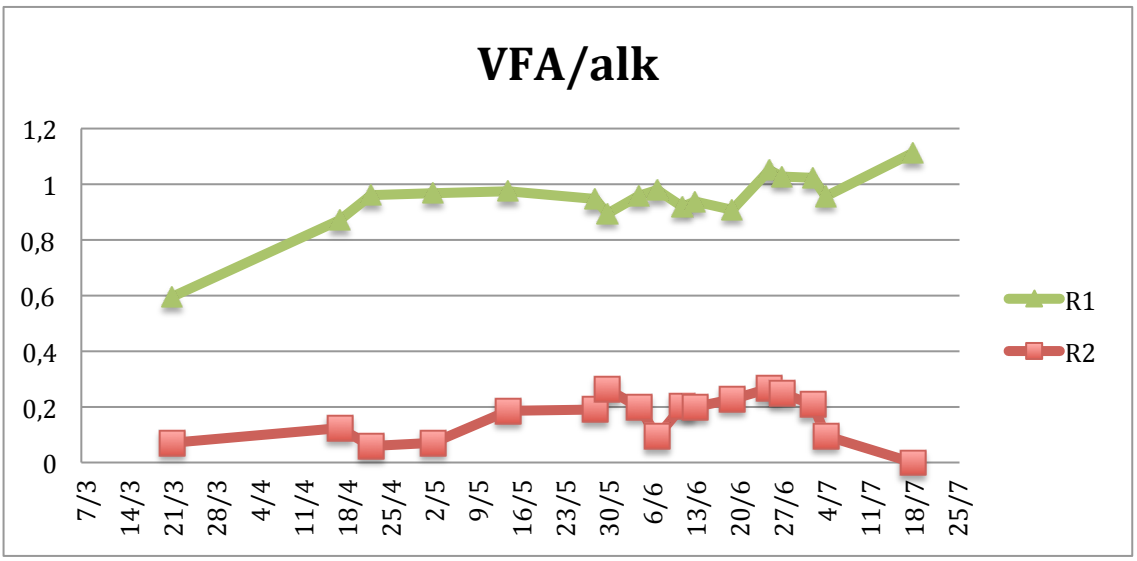

Fig 5.13: VFA/alk ratio for reactors R1 and R2

5.4 Conclusions

R1, a smaller reactor expressively meant to produce hydrogen and to provide feeding for R2, a second, methane-generating reactor, proved tthat two-stage AD is a process feisable in full scale. Hydrogen is produced and reaching concentrations over 30%, comparable to those found in lab scale tests; R2 showed to have a behaviour consistent with that of standard, on-stage AD full plants, thus not . Still, the full-scale posed a constant challenge. The project is expected to continue until 2015.

Where our current studies showed a chance on biohydrogen production, it was not possible to carry out the experiment longer due to technical issues.

Twice R1 experienced flowing problems caused by punt and pipes otturation; this led to an excess of pressure and in clefts in the reactor's walls. Gas leaks and lost of airtightness spoiled the process' dynamics: gas volume produced decreased (thus making it difficult for the blower pump to measure it) and hydrogen percentage dropped in favour of CO_2 and methane gas.

Much is still to be analyzed in order to understand wether a full-scale two-stage AD plant might have a role in future green energy production landscape; technical and engineering problems are to be analyzed, since they proved to be numerous and more frequently occurring than estimated.

Still, the evidence that lab-scale and full-scale processes proved a promising similarity, useful in order to foretell chemical and biological behaviour in a real plant.

5.5 Supporting information

Fig. 5.14: Upgrading station.

Fig. 5.15: Cogeneration plant.

5.16: *In situ*-produced biogas fueled van.

5.6 References

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6. CONCLUSIONS

This work aimed at analyzing the process of anaerobic digestion for the production of hydrogen. We started from a basic, laboratory scale and upgraded the project to reach, in the end, a full scale plant.

A first step consisted in a fast in batch series of tests in order to evaluate if and how the chemical composition ofthe various agricultural and waste biomasses used as alimentation was involved in hydrogen production. Thanks to a successful statistical approach we were able to determine that H2 production is a process positively influenced by highly degradable compounds (sugars and starch) while high levels of more recalcitrant molecules (e.g. lignine) inhibit fermentation.

The study proceeded then to more realistical representations. First, a semi-continuous test was carried out on four vegetal biomasses mixed with pig sludge at different concentrations, followed by a in batch single stage and second stage test for the same feeding mixtures. This test proved again that not all biomasses are suitable for two-stage anaerobic digestion: olive pomace, for example, produced no hydrogen. The two-stage process, at this step, showed better performances in specific conditions of hydraulic retention time and concentration when compared to the single stage.

A further step forward involved data coming from the previous test: the best performing experimental point (biomass, HRT and concentration) was replicated in a completely in continuous test. This better simulated the functioninf of a full scale plant: three reactors (a single stage one and a two-stage system) were fed with a mixture of rice middlings and swine manure. Output from single and second stage were later processed and used as fuel for microbial fuel cells (MFC). If the mere fermentative process showed no significative difference between classical, single stage AD and two-stage process, the creation of a third stage through MFCs showed promising perspectives and an higher energy production from the overall two-stage process.

Lastly, data and findings from the first experiments were applied oto the first european full-scale

plant. In spite of the many, different issues met during the start-up phase (especially technical and mechanical problems), there is a clear evidence that also a full scale reactor, when correctly taken care of and alimentated, allows to produce a biogas with an hydrogen content comparable to a lab scale simulation. While there is still much work to be done on the subject, it is interesting to note that an encouraging hydrogen production does not seem to affect the possibility to produce methane in a second stage. This simple differentiation in fuels is itself a promising improvement of the anaerobic digestion technology.

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