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"Bio-hydrogen production by self fermentation of vegetable waste: from screening of microbial diversity to bioaugmentation of indigenous fermentative communities."

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A Emiliana perché seppur temporalmente
dislocato questo traguardo è condiviso

A Francesco perchè la vita continua anche
se a volte è difficile crederlo

PREFACE

This thesis is the result of a PhD study conducted at the Department of Renewable Energy Sources, Laboratory of Biomass and Bioenergy, of the Italian Agency for New Technologies, Energy and Sustainable Development (ENEA).

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The research adopted an ecological and innovative approach for the study of fermentative hydrogen production from vegetable waste. Chapter 1 is a dissertation providing background to contextualize the study in a perspective of sustainable development for the establishment of a sustainable bio-based society and for understanding the important aspects of the dark hydrogen fermentation process. Each of Chapters 2 to 4 is consistent, in all its parts, with the manuscript submitted to a peer-reviewed scientific journal, for evaluation and possible publication. Chapter 5 contain the conclusion and final remark of the entire work. The last section of the thesis contains the literature cited in Chapters 1 and 5, while for Chapters 2, 3 and 4, the bibliographic references are provided separately at the end of each chapter.

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1. INTRODUCTION

The goal of our society is (should be) to build a new economy: an Eco-economy, which is by definition an economy that respects the principles of Ecology (Brown 2001).
According to the WCED (World Commission on Environment and Development), sustainable development is defined as "development that meets the needs of the present without compromising the ability of future generations to meet their own needs." (WCED 1987). It involves the maintenance of a delicate balance between the human need to improve lifestyles and well-being on one hand, and on the other preserving natural resources and ecosystems, on which we and future generations depend. This means that Sustainable development requires an assessment of the degree to which the natural resources of the planet are both present in sufficient quantity and in an accessible state to meet these needs, and to be able to deal with the wastes that we inevitably produce in manipulating these resources. Moving towards sustainability is possible only through a reduction of environmental burden which can be obtained along two routes: reduction of resources used per person and replacement of current raw materials, including energy (Clark and Deswarte 2008). The first route, although the most immediate, is the most difficult to follow since increasing wealth has brought with it an increase in levels of consumption. The second route, with the goal of sustainable development, represents a more fundamental approach to the problem, which requires to switch consumption to only renewable resources.
An ideal renewable resource is a natural resource that can be replenished naturally over a relatively short timescale or that is essentially inexhaustible. Resources such as coal, natural gas and crude oil exist in limited supply, as they come from organic carbon generated through photosynthesis many millions of years ago. They cannot be replenished by natural means at the same rates that they are consumed and thus are non-renewable. In contrast, resources such as solar radiation, winds, tides and biomass can be considered as renewable resources, which are (if appropriately managed) in no danger of being over-exploited. It is worthy of note that, uniquely among these renewable sources of energy, biomass can be used to produce also chemicals and materials. Nowadays governments and scientific institutions realize that waste and

lignocellulosic materials (e.g. residues from agriculture, forestry and industry) represent a better opportunity for exploitation, since they avoid competition with the food sector.
In order to maintain the world population in terms of food, fuel and organic chemicals, and tackle global warming, it has been recognized by a number of governments that we need to substantially reduce our dependence on petroleum feedstock by establishing a bio-based economy (van Dam *et al.*, 2005).

1.1 The Biorefinery: biotechnology for bioenergy production

Alternative production chains are called for in order to reduce the dependence on oil, to mitigate climate change originated by human activities and to redirect the current production processes (open-cycles) toward natural closed ecological cycles. Such alternative production chains necessitate a replacement of oil with biomass. It will require some breakthrough changes in today's production of goods and services: although biological and chemical science will continue to play essential roles in the generation of future industries, new synergies of ecological, biological, physical, chemical and technical sciences must be developed (Kamm et al. 2006). Accordingly, the aim of research is in developing new technologies and creating novel processes, products, and capabilities to ensure a growth that is sustainable from economic, environmental and social perspectives (Cherubini 2010).
Environmental biotechnology is the leading science in the effort to eliminate polluting compounds from waste streams, to generate a liquid, gaseous or solid residue that can be reused in a natural environment without detrimental ecological effects. The increasing interest in the effective use of natural resources suggests the addition of a second objective: the recovery of specific nutrients (e.g. nitrogen, phosphorous or sulfur) or the production of specific products (e.g. biofuels, organic solvents, bioplastics). This objective changes the status of several streams generated in the agro-industry from waste to raw material for the production of specific chemicals or energy carriers (Kleerebezem and Van Loosdrecht 2007). This implies the development of a new biotechnological research field that leads to diversified biomass utilization, comprised in the concept of a "biorefinery": such a system combines traditional

elements from environmental biotechnology in terms of cleaning of waste streams, with industrial biotechnology in terms of product maximization.

Similarly to oil-based refineries, where many energy and chemical products are produced from crude oil, biorefineries will produce many different industrial products from biomass. These will include low-value, high-volume products, such as fuels, commodity chemicals, as well as materials, and high-value, low-volume products or specialty chemicals, such as cosmetics or nutraceuticals (Fig. 1). Energy is the driver for developments in this area, but as biorefineries become more and more sophisticated with time, other products will be developed (Clark and Deswarte 2008).

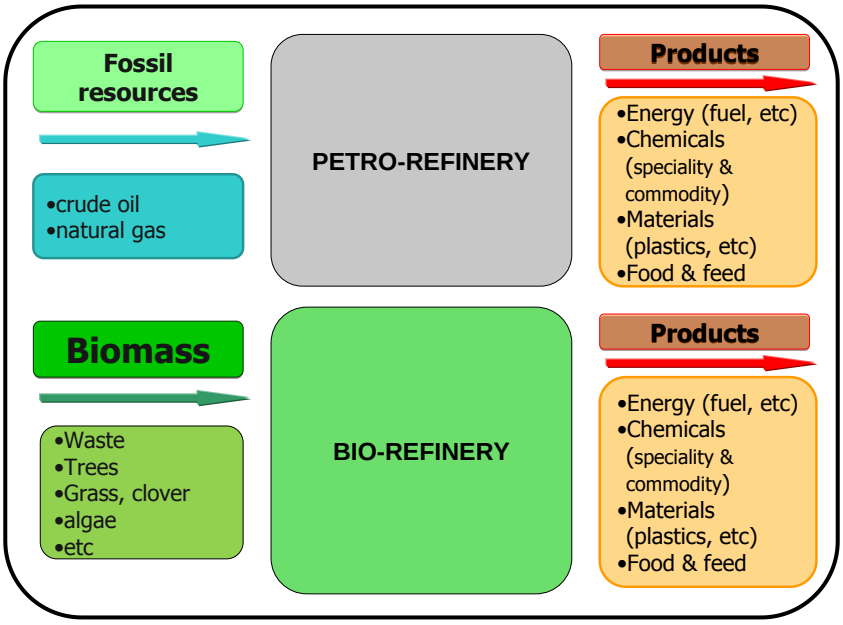

Figure 1. Comparison of petrorefinery vs biorefinery.

Owing to the use of natural microorganisms, the required metabolic capacities and the corresponding microbial population can be effectively enriched from a natural environment on the basis of natural/ecological selection by manipulating the bioprocess or by varying the source of the natural inocula.

At regional, national and global levels there are four main drivers for using biomass in a biorefinery for production of bioenergy, biofuels and biochemicals. These are climate

change, energy security, rural development and sustainable waste management. In order to overcome these issues, production of second-generation biofuels (i.e. from raw materials based on waste, residues or non-food crop biomass) gained an increasing worldwide interest in the last few years as a ‘‘greener” alternative to fossil fuels and conventional first-generation biofuels (usually referring to biofuels produced from raw materials in competition with food and feed industries). In the development of 2nd generation biofuel production, the use of locally available biomass resouces in biorefinery complexes is expected to ensure additional environmental benefits and implement national energy security, thanks to the coproduction of both bioenergy and high-value chemicals (Cherubini 2010).

Depending on the use of different types of feedstock, conversion technologies and products, a lot of biorefinery pathways, from feedstock to products, can be established giving rise to integrated bio-industrial systems in which the residue from one bio-industry becomes an input for other industries. Thus, the bio-refinery approach proposes a potentially complete exploitation of waste biomass, and many technological processes are jointly applied to different kinds of biomass feedstock for maximizing energy and material recovery. It represents one of the most important steps towards converting the current production of goods and services based on fossil resources into a new economy/society based on renewable biological raw materials, having bioenergy, biofuels and bio-based products as main pillars.

Through the development of biorefinery systems, production processes are directed towards closed systems that maximize recycling of materials and reproduce the natural ecological cycles, in order to lead to the development of a *'Society based on natural closed ecological cycles'*.

1.2 Anaerobic digestion

Anaerobic digestion is the classical example of a process that combines the objectives of sustainable waste management (Hartmann and Ahring, 2005; Lema and Omil, 2001) with pollution control and with the generation of a valuable energy product. This process offers the advantage of both a net energy gain as well as the production of

fertilizer from the residuals, allowing the recirculation of nutrients back to the soil (Edelmann et al., 2000; Sonesson, 2000), and contributing to reduce waste volume and costs for waste disposal. It involves the bacterial breakdown of biodegradable organic material in the absence of oxygen over a temperature range from about 30 to 65 °C (Figure 2 shows the stages of anaerobic digestion process). In the absence of an external electron acceptor, organic substrates can only be fermented; a process where the organic substrate is both electron donor and acceptor. The main end product of these processes is biogas (a gas mixture made of methane, CO_2 and other impurities), which can be upgraded to >97% methane content and used as a surrogate of natural gas (Romano and Zhang, 2008). Methane is the organic compound with the lowest free energy content per electron upon oxidation to carbon dioxide (Hanselmann, 1991). This indicates that in a thermodynamically closed system microorganisms can obtain the energy required for growth by catalyzing (stepwise) the conversion of organic substrates to methane and carbon dioxide.

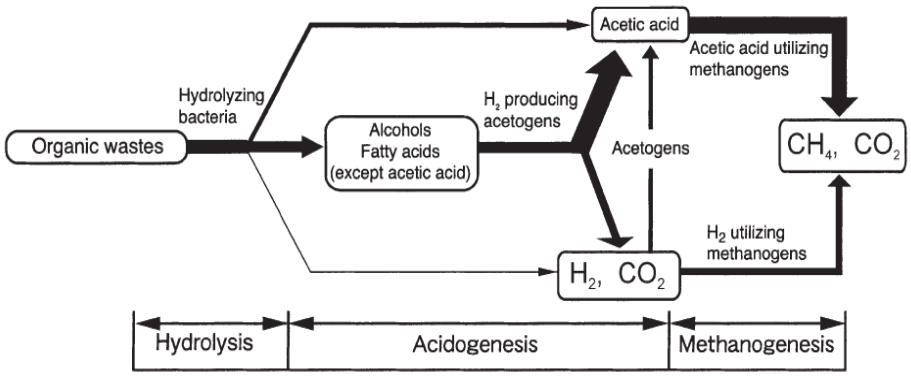

Figure 2. Stages of the anaerobic digestion.

Three main advantages favour anaerobic treatment compared to aerobic degradation of organic substrates: 1) the high product and low biomass yield resulting in a limited generation of waste sludge as an unwanted side product; 2) the in situ separation of the product as biogas, limiting costs for product separation; 3) the use of simple technology, as mixing by the biogas produced, circumvents the need for other mixing requirements.

Anaerobic digestion is a consolidated technology and at the present represents the best-suited option for bioenergy production from sludges, liquids and wet organic materials, even if its economic viability depends heavily on the availability of low-cost raw materials. Thus, it is generally based on the treatment of substrates like activated sludge, manure and concentrated industrial waste (Alatriste-Mondragon et al., 2006).

From a bioenergy concept point of view, the biotechnological production of methane has limitations owing to the low price of natural gas. In cases where a network for natural gas is available, biogas can be distributed through this existing infrastructure; however, carbon dioxide and hydrogen sulfide need to be removed from the biogas (Janssen et al., 1998).

Further advances in the field of anaerobic digestion of complex substrates are limited, owing to the rate-limiting hydrolysis process for solubilization of the particulate substrates. Enhanced degradation rates can only be established using generally expensive pretreatment methods (Kleerebezem and van Loosdrecht 2007).

However, thanks to the feed and flexibility of the products' end uses, anaerobic digestion plants are available for decentralized power generation and for creating multifunctional companies and bio-refineries. In fact, anaerobic digestion products can be used in many ways: biogas is available for cogeneration, for heat production or as vehicle fuel, and the digestate is available for agronomical uses or recycle of nutrients (Massi, 2012).

1.3 Biohydrogen production by fermentation

One possible successor to the fossil fuel era is the establishment of a hydrogen society, where hydrogen produced from renewable sources is the primary energy carrier. The two most important challenges are to generate enough hydrogen from renewable sources and to lower the production cost of hydrogen (Westermann et al., 2007).

Biohydrogen production by fermentation of organic wastes has received significant attention in recent years, since it not only treats organic wastes but also produces very clean energy (Das and Veziroglu, 2001; Hawkes, 2007; Das, 2009; . Hallenbeck, 2009).

The main driving forces for investigating the production of hydrogen instead of methane are the higher economic value of hydrogen, owing to its wider range of applications in the chemical industry (Lee and Fang, 2007) and greenhouse gases reduction. In fact, the major advantage of energy from hydrogen is the absence of polluting emissions since the utilization of hydrogen, either via combustion or via fuel cells, results in pure water (Kapdan and Kargi, 2006). It also has the highest energy content per unit weight of any known fuel (142 kJ/g) and can be transported for domestic/industrial consumption through conventional means (Benemann, 1996).

Under anaerobic conditions, hydrogen is produced as a by-product during conversion of organic wastes into organic acids which are then used for methane generation. This acidogenic phase of anaerobic digestion can be manipulated to improve hydrogen production.

Traditionally, the development of biological processes to transform biomass to more versatile energy carriers has focused on the production of methane as it is the only energy carrier released from the conversion of organic matter in nature.

Hydrogen is never released since in nature there are numerous other bacteria, which readily consume hydrogen as a source of reducing power, therefore hydrogen is an intermediate during anaerobic degradation and is further metabolized to methane.

In natural environments, microbial activity is governed by an ecological niche. This niche is the resultant of many contributing factors including the presence and quality of available organic matter, presence of minerals, temperature, light, pH, salinity, redox potential, synergistic or antagonistic activity of microbial populations etc. The ecological niche governs the activity of certain microbial populations and thus the concentration and variety of the final products.

When the aim is H_2 production a physical separation is required of individual processes in the anaerobic degradation chain (this can be carried out in a biorefinery), or the creation of a specific environment in which hydrogen producing microorganisms flourish and others perish.

Therefore, successful biological hydrogen production requires inhibition of hydrogen-using microorganisms, such as homoacetogens and methanogens. Inhibition is commonly accomplished by heat treatment of the inoculum to kill all microorganisms except for spore-forming fermenting bacteria. Other methods that have been used

include the control of operation parameters of reactors: high dilution rates, low pH, low hydrogen partial pressure, nitrogen gas sparging (de Vrije and Claassen, 2003).

The main limitation of hydrogen production by fermentative bacteria is the maximum electron-based yield that can be established. Owing to biochemical and thermodynamic limitations, the maximum theoretical number of moles of hydrogen that can be generated per mole of glucose is four, and can be obtained by glucose oxidation to two moles of acetate. (Complete oxidation to carbon dioxide and hydrogen, would produce 12 moles of hydrogen per mole hexose, however, is never attained in known biological *in vivo* systems due to thermodynamic constraints). Actual yields are even lower than the four moles of hydrogen that are theoretically possible, typically ranging from 1 to 2.5 moles hydrogen per mole hexose.

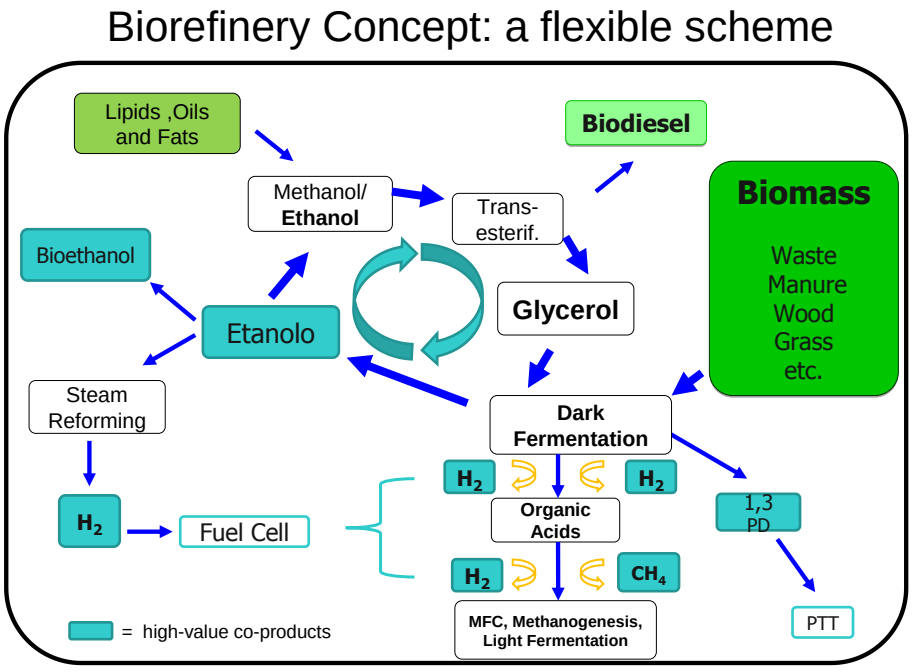

Figure 3. A possible example of bio-hydrogen refineries.

Thus, fermentative hydrogen production provides only a partial oxidation of the organic substrate and is therefore normally integrated in a two-step process. In such a process, the fermentation products (volatile fatty acids) are either converted to methane-

containing biogas or converted to hydrogen and carbon dioxide in a phototrophic process. Alternatively the products of the fermentation process can be used to produce electric energy in a Microbial fuel cell (MFC) or further H_2 can be obtained in a bioelectrochemically assisted microbial reactor (BEAMR), in which hydrogen is evolved at the cathode by eliminating oxygen at the cathode, and adding a small voltage to the circuit. Therefore, direct biological production of hydrogen through dark fermentation could be considered a primary step in a larger bioenergy or biochemical production concept (Fig. 3).

1.3.1 *Physiology of dark hydrogen fermentation*

Dark fermentative H_2 production is a ubiquitous phenomenon that occurs in natural environment under anoxic or anaerobic conditions. It consist of obligate series of microbial complex biochemical reactions occurring under anaerobic conditions when electron acceptors (O_2, Mn^{4+}, NO^{-3}, Fe^{3+},SO_4^{2-}, HCO_3^-) are absent or negligible. The organisms are capable of an amazing array of different types of fermentation as they are characterized by great metabolic versatility, both among species and within the same species or strain. Fermentation can involve any molecule that can undergo oxidation. Typical substrates include sugars (such as glucose) and aminoacids.

Micro-organisms derive energy from substrate-level phosphorylation: a part of the substrate carbon is oxidized and another part is reduced leading to the formation of products that are more or less oxidized than the original substrate. Electrons derived from this process are used for the reduction of protons to molecular H_2.

Molecular hydrogen formation generally follows two routes in the presence of specific coenzymes, either by the formic acid decomposition pathway or by the re-oxidization of nicotinamide adenine dinucleotide (NADH) pathway (Nandi and Sengupta, 1998; Hallenbeck and Benemann, 2002; Hallenbeck, 2005). H_2 evolution per se does not confer any advantage to microbes, however it is obligatory for the elimination of excess electrons for some members of the microbial community.

In all thermodynamically feasible dark fermentation processes exploited by known microorganisms, H_2 is only produced in combination with volatile fatty acids (VFA) and/or alcohols—never as the single reduced compound. There are several types of

metabolic pathways and between classes of organisms there is some variability as to which of these pathways are viable.

In all the cases glucose is a key compound in microbial metabolism. It is first converted to pyruvate, producing adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and the reduced form of nicotinamide adenine dinucleotide (NADH) via the glycolytic pathway [Eq 1].

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3COCOOH + 2NADH + 2H^+ \quad (1)$$

Pyruvate is then further converted to acetylcoenzyme A(acetyl-CoA), carbon dioxide, and H_2 by pyruvate–ferredoxin oxidoreductase and hydrogenase. Pyruvate may also be converted to acetyl-CoA and formate. Acetyl-CoA is finally converted into acetate, butyrate, and ethanol, depending on the microorganisms and the environmental conditions.

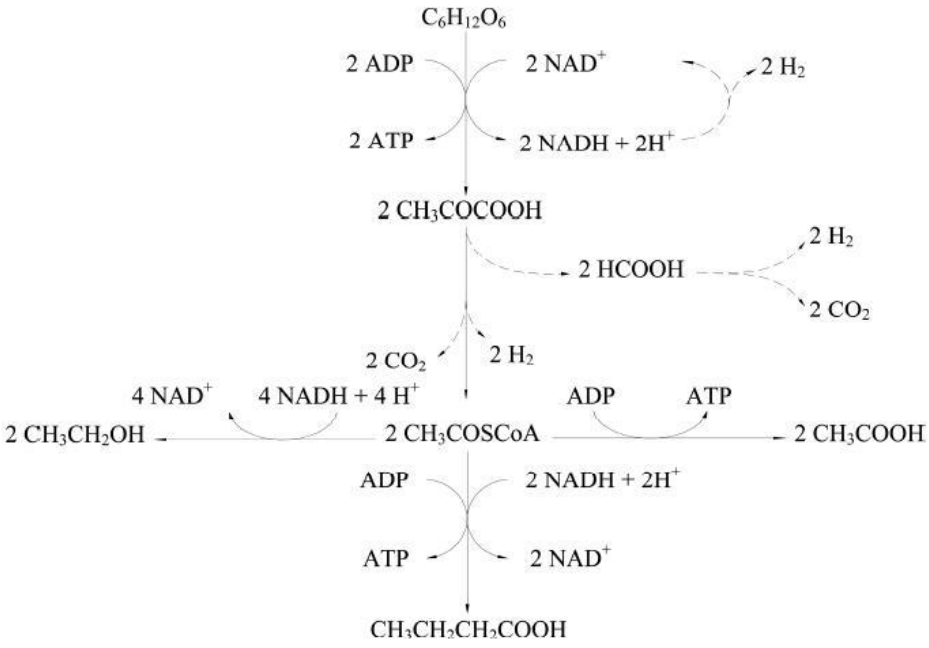

Figure 4. Pathway of hydrogen production from fermentation of glucose (Yan et al., 1988; Prescott et al., 2002).

The excess of NADH generated during glycolysis is a limiting factor of the fermentation process, it prevents further oxidation of substrate due to a lack of an NAD^+ pool to accept electrons so that glycolysis and oxidative decarboxylation of pyruvate can proceed to generate ATP. In many fermentation pathways, the steps after energy generation are performed in part to regenerate the pool of NAD^+. The means of regenerating NAD^+ include the formation of H_2 and/or reduced products such as lactate, ethanol, butyrate, succinate and propionate (Stams, 1994), with, in some cases (acetate, butyrate and succinate), concomitant ATP production through substrate level phosphorylation (Madigan, 2000).

Many pathways have been proposed for H_2 production using glucose as the model substrate as shown in figure 4.

The most common products in the fermentation of carbohydrate are acetate, butyrate and ethanol [Eqs (2), (3) and (4)]. The H_2–acetate couple produces more ATP per mol of substrate than alcohols such as ethanol and butanol and is the energetically ‘‘preferred’’ bacterial fermentation product for a sugar (Logan, 2002).

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

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (3)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2H_2 + 2CO_2 \quad (4)$$

According to reaction (2-4), the stoichiometric yields are 4 moles of H_2 for each mole of glucose in the production of acetic acid (Nandi and Sengupta, 1998) and 2 moles of H_2 in the production of butyric acid (Nandi and Sengupta, 1998) or ethanol (Gaudy and Gaudy, 1980; Hwang et al., 2003). However maximum H_2 yield from fermentative H_2 production can be achieved when only volatile fatty acids (VFAs) are produced and no microbial growth occurs (Angenent et al., 2004). Current H_2 productivities are in the range of 10–20% (Kalia et Purohit 2008), which is equivalent to 1.17 to 2.34 mol H_2/mol glucose (Angenent et al., 2004; Beneman, 1996; Logan, 2004). Over the energy involved in biomass production, there are several reasons for the low actual H_2 yield: glucose may be degraded through other pathways without producing H_2; a

stoichiometric yield is achievable only under near equilibrium condition, which implies a slow production rate and a low H_2 partial pressure (Woodward et al., 2000; Hallenbeck and Benemann, 2002); some H_2 produced may be consumed for the production of other by-products, such as propionate (Vavilin et al., 1995).

1.3.2 *Hydrogen producing microorganisms*

Diverse microbes capable of H_2 production by dark fermentation are distributed across a wide variety of bacterial groups (Lee et al., 2011). The organisms used in dark fermentation studies include anaerobes, facultative anaerobes and aerobes in a wide temperature range (mesophiles, thermophiles and hyperthermophiles). Mesophiles are mainly affiliated with two genera: facultative Enterobactericeae (Kumar and Das, 2000) and strictly anaerobic Clostridiaceae (Collet et al., 2004; Evvyernie et al., 2001; Wang et al., 2003), whereas most thermophiles belong to genus Thermoanaerobacterium (Ahn et al., 2005; Ueno et al., 2001; Zhang et al., 2003). Also few aerobes, such as Bacillus (Kalia et al., 1994; Kumar et al., 1995; Shin et al., 2004), Aeromonons spp., Pseudomonos spp. and Vibrio spp. (Oh et al., 2003b) have been characterized for H_2 production under anaerobic conditions but they show H_2 yields less than 1.2 mol-H_2/mol-glucose.

Clostridia are strict anaerobes, Gram-positive, rod-shaped, extremely sensitive to oxygen so that the presence of a trace amount of dissolved oxygen can completely stop the H_2 production (Chen et al., 2002). Hence, clostridial growth media are usually supplemented with a reducing agent to ensure anaerobiosis. In addition, Clostridia may form endospores, a ‘‘survival mechanism’’ developed by these organisms when unfavorable environmental conditions are encountered (e.g., high temperature, desiccation, lack of carbon or nitrogen source, chemical toxicity). This requires close monitoring of feeding rate and of operating parameters of the reactors to minimize sporulation (Hawkes et al., 2002). At the same time the ability of Clostridia to form spores, is widely exploited in dark fermentation studies as it allows to easily select such bacteria, among natural microbial community, simply applying thermal and/or chemical treatments to the substrates and/or inocula. These treatments at the same time are used to eliminate the H_2-consuming, non-spore-forming organisms.

Clostridia lack a typical cytochrome system and obtain energy by substrate-level phosphorylation during fermentation. Oxidizing carbohydrates generates electrons which need to be disposed of to maintain electrical neutrality. The breakdown of pyruvate to acetyl-CoA and CO_2 by means of pyruvated ferrodoxin oxidoreductase, (PFOR) produce 2 mol of reduced ferredoxin [Eq. 5]. The reduced ferredoxin transfers electrons to a [FeFe] hydrogenase, driving H_2 evolution [Eq. 6]. This assures the production of two moles of H_2 per mole of glucose consumed.

Clostridia possess a pathway that allows them, under the proper conditions (low pH_2) to produce additional H_2 by reoxidizing the NADH generated during glycolysis: the NADH can transfer its electron to ferredoxin via NADH Ferrodoxin oxidoreductase (NFOR).

$$NADH + H^+ + 2Fd^{2+} \rightarrow 2H^+ + NAD^+ + 2Fd^+ \quad (5)$$

$$2Fd^+ 2H^+ \xrightarrow{hydrogenase} 2Fd^{2+} + H_2 \quad (6)$$

Since two moles of NADH are produced during glycolysis of glucose, two additional molecules of H_2 could potentially be generated by this pathway. Thus, these organisms appear to be capable of producing up to 4 mol H_2/mol glucose which is the theoretical maximum yield of dark hydrogen fermentation if all of the substrate would be converted to acetic acid (Thauer, 1977). If all the substrate would be converted to butyric acid, this value is 2 moles of H_2 per mole of glucose, since 2 mol of NADH has been consumed during the conversion of intermediate products. The available H_2 from glucose fermentation is determined by the ratio of butyrate/acetate produced during fermentation if no NADH is used as a reductant for alcohol production.

The actual optimum H_2 yields observed vary between 1.1 mol-H_2/mol-hexose and 2.6 mol- H_2/mol-hexose, dependent on the organism per se as well as environmental conditions (Lee et al., 2011). The explanation for these lower yields is due to the fact that the disposal of electrons via NFOR and hydrogenase might be affected by the corresponding NADH and acetyl-CoA levels as well as environmental conditions. In addition, proton reduction with NADH is unfavourable since the redox potential of H_2 (~450 mV at neutral pH) is appreciably lower than that of the NADH/NAD couple

(~320 mV). Thus, production of H_2 with electrons derived from NADH is only possible at greatly reduced pH_2 (Kataoka et al., 1997; Mizuno et al., 2000). The inability to reoxidize NADH by this latter pathway leads to its oxidation along pathways that produce reduced organic compounds; i.e., lactate, ethanol and butanol, resulting in a lowered H_2 yield. (Figure 5 shows the glucose oxidation pathway of *Clostridium butyricum*.)

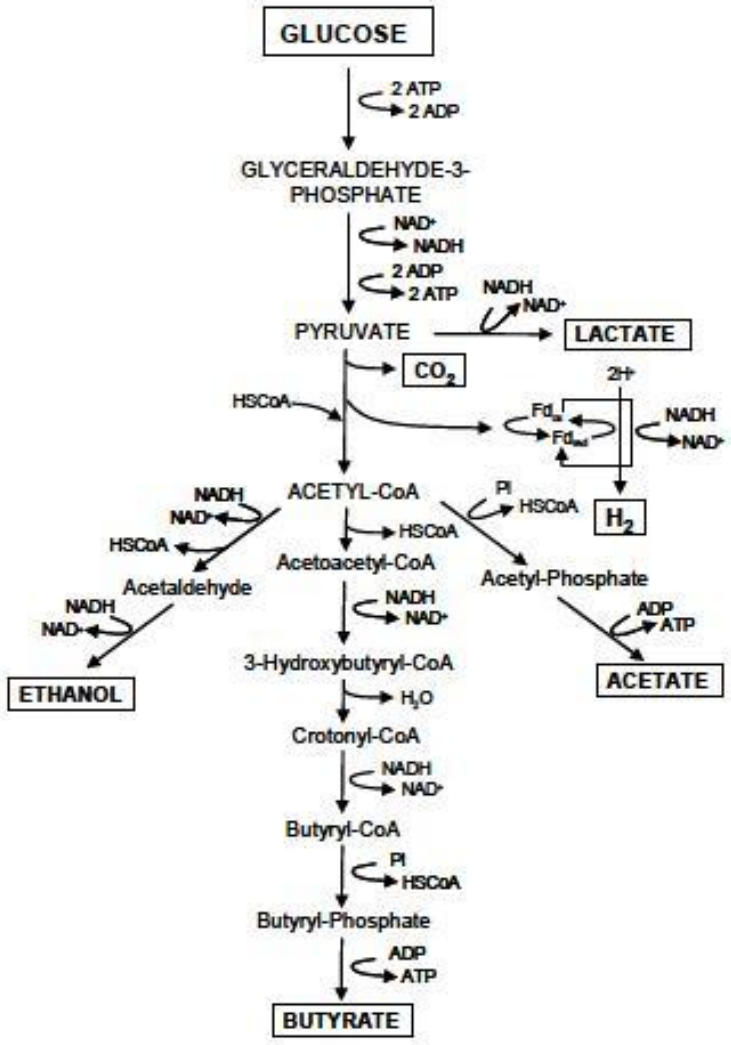

Figure 5. Clostridial-type hydrogen producing fermentation (Modified from Saint-Amans et al. 2001).

Enteric bacteria are facultatively anaerobic, Gram-negative, non-sporulatig rod-shaped, they can be grown more easily than obligate ones, since they tolerate oxygen (Madigan et al., 2000).

They express a mixed acid fermentation type in which H_2 is generated by formate degradation (sometimes called formic acid fermentation) catalized by H_2 lyase (FHL) complex (comprising a specific formate dehydrogenase and hydrogenase).

Pyruvate is first cleaved to acetyl-CoA and formic acid by pyruvate-formate lyase with the production of ATP via acetyl phosphate and without the immediate production of reducing equivalent. Under anaerobic conditions and in the absence of suitable electron acceptors, the formic acid will be further degraded to H_2 and carbon dioxides via formic hydrogenlyase [Eqs. (7) and (8)].

$$CH_3COCOOH + H\ CoA \rightarrow CH_3COCoA + HCOOH \quad (7)$$

$$HCOOH \rightarrow H_2 + CO_2 \quad (8)$$

As with the Clostridia, Enterobacteriaceae have to dispose of excess reductant produced during fermentation, and this is accomplished by the eventual production of H_2.

It is noted, therefore, that NADH produced during anaerobic glycolysis is rarely used for H_2 production by the bacteria of genus Enterobacteriaceae, due to the absence of specific coenzymes such as ferredoxin oxidoreductase. Nevertheless, NADH must still be oxidized back to NAD+, otherwise anaerobic glycolysis will cease.

Many microorganisms solve this problem by slowing or stopping pyruvate dehydrogenase activity and using pyruvate or one of its derivatives as an electron and H_2 acceptor for the re-oxidization of NADH in a fermentation process. As a result, the oxidation–reduction state has to be balanced through the NADH consumption to form a large amount of mixed acids and alcohols, most of which are the H_2-containing reduced products, and which are accompanied by the formation of formate. Proportions of reduced products, principally ethanol, and acetate vary in order to maximize ATP production while at the same time reoxidizing NADH to provide the NAD needed by the glycolytic pathway for further substrate utilization and change depending upon the redox state of the substrate and the pH. Lactate formation and formate degradation to

CO_2 and H_2 are induced at acid pHs which serve to relieve the acid stress. (Figure 6 shows the glucose oxidation pathway of *Escherichia coli.*). Production of these reduced compounds limits H_2 release in a gas form and results in a lowered H_2 yield which is generally less than 2 mol-H_2/mol-glucose by facultative anaerobes compared with anaerobic Clostridia.

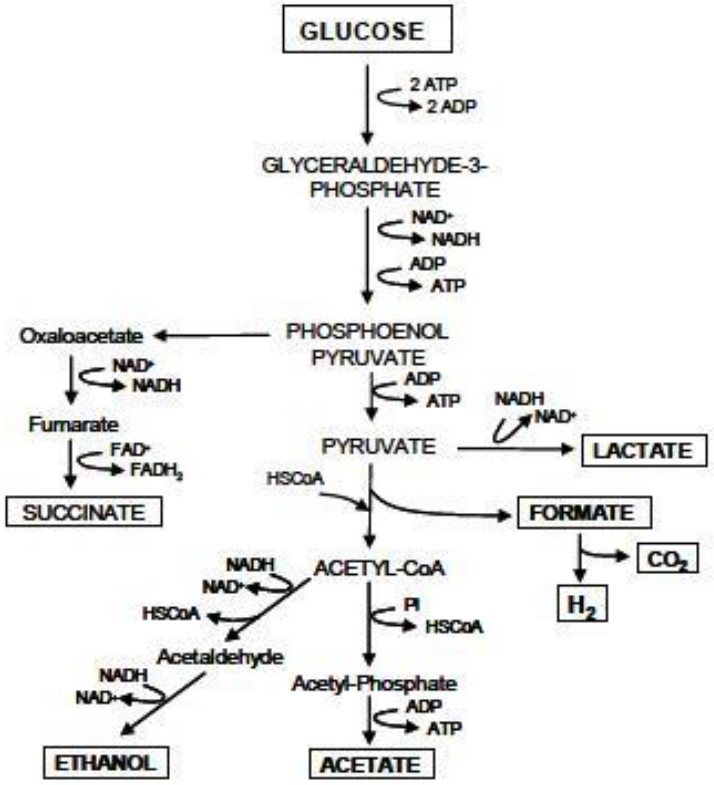

Figure 6. Enteric-type mixed-acid fermentation (Modified from Turcot et al. 2008).

Rather than for these low yields, the use of facultative anaerobes together with obligate in H_2 fermentation process is beneficial since facultative anaerobes might accelerate the reactor startup by creating an anaerobic environment for the O_2-sensitive Clostridia, and thus avoid the addiction of reducing agents in the medium (Yokoi et al., 1998).

Hyperthermophilic cultures are capable of H_2 production with higher yields than mesophiles (Hallenbeck, 2005), the highest H_2 yields reported so far, are close to the

theoretical maximum of 4.0 mol-H_2/mol glucose which was achieved by using extreme thermophiles (Schröder et al., 1994). In addition, several thermophiles and hyperthermophiles have the capacity to utilise complex carbohydrates such as cellulose and starch, a valuable property widening the potential for the use of industrial waste streams and agricultural residues as feeds.

Thermophilic cultures are resistant to overgrowth by mesophilic contaminants but, although an economic analysis is not available, the energetic costs associated with maintaining 70°C may be mitigated with large-scale application (Redwood et al., 2009).

The necessity of growth on solid media for molecular work (i.e. at temperatures lower than the melting point of agar), makes hyperthermophiles less readily amenable to genetic engineering, although alternative solid media such as gelrite are available (Van Ooteghem et al., 2004). In addition, comparing with mesophilic cultures, hyperthermophiles exhibit much lower production rates of H_2, generally ranging from 0.01 to 0.2 LH_2/L_h, which is largely attributed to the slow-growing characteristics of hyperthermophiles (Lee et al., 2011).

Little biochemical information is as yet available (de Vrije et al., 2007) but the biochemical reactions involved in thermophilic H_2 production seem different from those of mesophiles. Observations support the connection of H_2 production with the hydrogenase-linked oxidation of electron carriers (as in clostridia), rather than the decomposition of formate (as in enteric bacteria).

Schröder et al. (1994), proposed a metabolic route based on glucose fermentation in growing cultures of hyperthermophilic Thermotoga maritime. Acetate is found to be produced as the unique soluble metabolite from the pyruvate, whereas H_2 and carbon dioxide are gaseous products. The enzymes involved in H_2 formation (NADH: ferredoxin oxidoreductase and hydrogenase) are typical for anaerobic bacteria, such as Clostridia, indicating that the pathway of re-oxidization of NADH is followed to form H_2. Another metabolic pathway involves the production of a reduced end product, L-alanine, together with the acetate (acetate–alanine pathway) (Kanai et al., 2005). Alanine is formed by alanine aminotransferase directly from pyruvate via transamination with glutamate, and it can be hypothesized that the generation of H_2 and alanine are competitive means of disposing intracellular reducing equivalents.

1.4 Aims of this work

The main objective of "Biological Hydrogen Production" is the development of a feasible process for hydrogen production from biowaste by micro-organisms.

The experimental hypothesis of this PhD study originates from the idea of considering the waste itself as a natural ecosystem with a complex microbial community which solve a complete series of ecosystem functions including carbon and nutrient cycling. This community theoretically could have, of itself, the microbial metabolic capacities required for hydrogen generation.

Therefore, manipulation of the background conditions to create an ecological niche suitable for hydrogen production could be an effective strategy to improve this process within the waste itself. In addition, the study of natural H_2-producing micro-organisms contained in the waste could allow us to tap into a great genetic diversity and select specialized organisms suitable for enhancing the hydrogen production functionality of the indigenous microbial community.

In this respect, this study has adopted an ecological and innovative approach for the development of a renewable and cost-effective process for fermentative H_2 production by:

- exploring the feasibility of H_2 production by mesophilic anaerobic self-fermentation of common domestic vegetable waste without pretreatment or added nutrients and improving the process by controlling operative parameters such as temperature and pH
- isolating and identifying potential H_2 producer bacterial strains contained in the waste and characterizing the fermentation metabolism of isolates using glucose as nutrient source
- performing bioaugmentation of the indigenous microbial community through the addition of pure culture and a bacterial consortium of three selected "indigenous" bacterial strains (the three single strains were also characterized for their ability to produce H_2 on different sugars, key products of hydrolysis of cellulose and hemicelluloses).

2. VEGETABLE WASTE AS SUBSTRATE AND SOURCE OF SUITABLE MICROFLORA FOR BIO-HYDROGEN PRODUCTION

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ABSTRACT

The aims of this study were: 1) to investigate the potentiality of bio-hydrogen self-fermentation process from leaf-shaped vegetable refuses (V) and leaf-shaped vegetable refuses plus potato peels (VP), under two mesophilic anaerobic conditions (28 and 37°C). The batch experiments were carried out without inoculum addition and pretreatments 2) to isolate and to identify potential H_2-producer bacterial strains contained in the vegetable extracts. The effect of initial glucose concentration (1-5-10 g/l) on fermentative H_2 production by the isolates was also evaluated.

H_2 production from self-fermentation of both biomasses was found to be feasible, without methane evolution, showing the highest yield for V biomass at 28°C (23.9 l/Kg VS). The pH control of the culture medium proved to be a critical parameter. The isolates had sequence similarities ≥ 98% with already known strains, one belonging to the family *Streptococcaceae* (*Firmicutes*) and ten to the family of *Enterobacteriaceae* (γ – *proteobacteria*). The found genera *Pectobacterium*, *Buttiauxella*, *Raoultella*, *Rahnella*, were not previously investigated for H_2 production. The isolates showed higher yield (1.58-2.19 mol H_2/mol glucose) at glucose concentration of 1 g/l. The maximum H_2 production ranged from 410-1016 ml/l and was obtained at a substrate concentration of 10 g/l.

Keywords: Self-fermentation; bio-hydrogen production; vegetable waste, facultative anaerobes; H_2 producing bacteria.

2.1 Introduction

As a sustainable energy carrier, hydrogen is considered to be the fuel of the future, mainly due to its high energy yield (122kJ/g), recyclability and non polluting nature. Among the methods for its production, the most renewable, sustainable and environmental friendly one seems to be the dark fermentation of organic wastes as it solves both the problem of clean energy production and waste disposal [1]. Organic waste decomposes by itself, if it is left for prolonged time at ambient temperature, because of the presence of abundant indigenous microflora, including hydrogen production bacteria [2]. Despite this, at present only few studies reported bio-hydrogen production from wastes self-fermentation. According to literature overview [2-21], biological hydrogen production by self-fermentation is defined as an heterotrophic anaerobic fermentation of waste without inoculum addition, regardless of whether the substrate/inoculum is subjected to chemical and physical treatments. In most of the published researches, heat pretreatments (including sterilization) of substrate/inocula were performed to improve hydrolysis process, to select H_2-producing community and to kill non spore-forming microorganism, mainly methanogens. However, these pretreatments eliminate also useful microorganisms, like facultative anaerobic H_2-producing bacteria that can promoting hydrogen production by maintaining a strict anaerobic environment and by breaking down complex substrates [22]. Ohnishi et al. (2010) [3] characterized a fermentative bacterial community of leaf litter cattle compost enable to produce hydrogen from food waste. Both the inoculum and the substrate were not subject to pretreatment or active control of anaerobic condition, The results showed that Mega*sphaera elsdenii* was the dominant H_2-producing bacterium and lactic acid-producing bacteria (LaB) were prevalent. In particular *Lactobacillus spp* consumed oxygen and produce lactate and *M. elsdenii*, an anaerobic non spore-forming, gram-negative bacterium produced hydrogen by using lactate. Both strains were unlikely to be introduced into H_2 fermentation systems using heat shock-pretreated inoculum. Bearing in mind that ideal reactor design should consider removing oxygen from feedstock before it enters the bioreactor, as much as possible, and considering the size of industrially feasible operations, the co-metabolism within the microbial community for

oxygen depletion was not only advantageous, but played a pivotal role in bio-hydrogen production [22].

On the other hand, in batch and in continuous mode, the natural selection of desirable native species can be carried out by controlling the reactor operating conditions such as pH, temperature, hydraulic retention time and organic loading rate [4-7,23]

So far, a few studies of H_2 production by self-fermentation without pretreatments, have been reported. To our knowledge, Yokoyama et al. (2007) [5] were the first authors that implemented this practice cultivating cow waste slurry at various temperature without inoculum addition and pretreatments. The highest yield of H_2 production (137.2 ml H_2/g VS) by self fermentation was obtained from food waste in thermophilic condition (50°C) [21]. However, Perera *et al.* (2010)[24] showed that negative net energy balance was achieved when the fermentation temperature exceeded 30°C.

In such context, this study has two objectives. First, to investigate the feasibility of biological H_2 production by dark self-fermentation of common domestic vegetable waste without lengthy and costly pretreatments. Two mesophilic temperature regimes were tested: below and above 30°C (28 and 37°C, respectively). The addition of chemicals was limited to buffer compounds to reduce the large pH fluctuations during the initial growth period [25] and to contrast the pH decrease due to the accumulation of acids during fermentation. In this view the efficiency of KH_2PO_4/Na_2HPO_4, 0.1 M, buffer was tested and the H_2 production performances were compared with those obtained without using buffer. Second, to explore the diversity of cultivable H_2-producing bacteria in waste extracts under mesophilic anaerobic conditions (28°C). The effect of initial glucose concentration (1-5-10 g/l) on fermentative H_2 production by the isolates was also evaluated.

2.2 Materials and Methods

2.2.1 *Feedstock preparation and culturing media*

Vegetable waste was collected from a cafeteria at the ENEA Casaccia Research Centre, Rome. Two different types of feedstocks were utilized: leaf-shaped vegetables refuses (V) and a mixture (wet mass) composed by 80% of leaf-shaped vegetables and 20% of potato peels (VP). The mixture composition reflected the average weekly production of

these wastes in the cafeteria. The total solid (TS) and the volatile solid (VS) of the substrates were estimated, according to standard methods [26].

Basal Fermentation Medium (BFM) was used as growth medium for isolation and batch H_2 production experiments. It consisted of (g/l): peptone 3; yeast extract 1; $FeSO_4$ $7H_2O$ 0.1; $MgCl_2$ 0.1; 10 ml mineral salt solution (g/l: $MnSO_4$ $7H_2O$ 0.01; $ZnSO_4$ $7H_2O$ 0.05; H_3BO_3 0.01; $CaCl_2$ $2H_2O$ 0.01; Na_2MoO_4 0.01; $CoCl_2$ $6H_2O$ 0.2; $AlK(SO_4)_2$ $\cdot 12H_2O$ 0.01; NiCl $6H_2O$ 0.001) and 5ml vitamin solution (g/l: cobalamin 0.01, vitamin C 0.025; riboflavin 0.025; citric acid 0.02; pyridoxal 0.05; folic acid 0.01; creatine 0.025) dissolved in 1l of KH_2PO_4-Na_2HPO_4 buffer (final concentration 0.1M pH 6.70) [27]. After sterilization of the medium (121°C for 15 min) mineral salts, vitamins and glucose, previously sterilized by filtering with 0,22 µm filter, were added. Agar 15 g/l, was added for isolation plates.

Preculture basal medium was used to revive the bacterial strains as reported in Pan et al.(2008)[27].

2.2.2 *H_2 production by self fermentation*

Batch experimental trials were carried out to examine H_2 production from non pretreated vegetable kitchen waste, using microflora naturally present within the waste. The experiments were carried out in 500 ml reactors, with a working volume of 250 ml, incubated anaerobically at 28 and 37°C, with magnetic stirring (120 rpm). The wastes, V and VP, were crumbled to less than 2 cm particle size and were diluted using 0.1 M phosphate buffer (KH_2PO_4–Na_2HPO_4) pH 6.70, and without buffer, using tap water, pH 7.00, for a final 0.4 w/v ratio. The experiments were performed in duplicate.

During fermentation the total amount of biogas produced (ml) and biogas composition (%) were determined. At the end of fermentation the composition of soluble products was analyzed

2.2.3 *Isolation and selection of H_2-producing bacteria*

The isolation was performed on two extracts obtained by homogenizing 1g of the samples V and VP in sterile saline solution (0.9% v/v). Serial dilutions of the two extracts were plated on agar BFM (glucose 10 g/l). Plates were incubated at 28 °C for 48h in an anaerobic jar filled with N_2 gas. Under anaerobic chamber single distinct

colonies, showing different morphologies, were picked up from plates of 10^3, 10^4, 10^5-fold dilutions. Each colony was re-streaked on fresh plates more than three times to ensure the purity of the isolates. Bacterial cells were picked up from the surface of BFM plates and aseptically transferred in 25 ml serum bottles containing 10 ml of BFM liquid medium (glucose 10 g/l). Cultures were incubated at 28 °C.

Bacterial isolates exhibiting H_2 production activity (≥ 0.2 mol H_2/ glucose) were selected for further studies and cryopreserved in glycerol (30%v/v) at -80°C until use.

2.2.4 *16S rRNA gene sequencing and analysis*

DNA of each selected bacterial isolate was prepared by lysis of 2-3 colonies grown overnight on BFM plates according to the procedure described by Vandamme et al. (2002) [28].

PCR amplification of the 16S rRNA gene was carried out using eubacterial universal primers 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CTACGGCTACCTTGTTACGA-3') with 2µl of each cell lysate suspension in 20µl of Quiagen Taq buffer with 0.5 U of Taq DNA polymerase, as reported by [29]. PCR products were purified using the Sephadex TM G-100 resin according to the supplier's instructions and quantitatively analyzed using the spectrometer Thermo Scientific NanoDropTM.

Sequencing reactions were prepared using the sequencing kit Applied Biosystem Big Dye Terminator ® version 3.1, according to the manufacturer's instructions, and analyzed with an ABI PRISM 310 Genetic Analyzer Perkin-Elmer, at the ENEA Genome Research Facility DNA Sequencing Laboratory (GENECHRON, ENEA C.R. Casaccia, Italy). Thermal cycling was performed with a gene Amp PCR System 9700 instrument (Applied Biosystems).

Sequence similarity searches were performed using the BLAST network service of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) and taxonomic analysis of 16S rRNA gene sequences was performed using the RDP Classification Algorithm (http://rdp.cme.msu.edu/classifier/classifier.jsp). Sequences obtained in this study and reference sequences obtained by BLAST analysis were aligned using CLUSTAL-W program (www.ebi.ac.uk/Tools/clustalw2). The resulting file was used for the construction of a phylogenetic tree using Neighbour Joining with Kimura 2 parameter

distances in MEGA software version 4 [30]. The robustness of the phylogeny was tested by bootstrap analysis with 1000 iterations.

The sequences generated in this study have been deposited in the Gen Bank database.

2.2.5 *Batch experiments for H_2 production from glucose using selected isolates*

Hydrogen production potential of the isolates was investigated. Batch dark fermentation experiments were carried out at three different initial glucose concentration (1, 5 and 10 g/l). The bacterial isolates were revived in pre-culture basal medium. After anaerobic incubation at 28°C with shaking at 120 rpm for 24h, the cultures were used as inocula. The H_2-production tests for each isolate were carried out in 125 ml serum bottles, containing 50 ml of BFM. The bottles, capped with rubber stopper and aluminum seals, were inoculated 1% (v/v) ratio and flushed for 2 min with filtered (0.22μm) pure nitrogen gas, to remove oxygen. The bottles were incubated at 28°C in a thermostat with orbital shaking of 120 rpm. The experiments were performed in duplicate.

During fermentation the total amount of biogas produced (ml) and biogas composition (%) were measaured. Hydrogen partial pressure in the headspace of batch culture was, also, calculated based on the volume of gas release from the reactor and the percentage of hydrogen in the biogas.

At the end of fermentation the composition of soluble products (VFA, lactate and ethanol) as well as glucose consumption were analyzed.

2.2.6 *Methodology*

Biogas production, was measured using water displacement equipment [31].

The volume of produced H_2 was calculated by the mass balance equation (1) [32]:

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_H\left(C_{H,i} - C_{H,i-1}\right) \quad (1)$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative H_2 gas volumes at the current (i) and previous (i–1) time intervals, respectively; $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at the current (i) and previous (i–1) time intervals; $C_{H,i}$ and $C_{H,i-1}$ are the fraction of H_2 gas in the headspace at the current (i) and previous (i–1) time intervals, and V_H is the total volume of headspace in the reactor. The cumulative H_2 production (H) data were fitted to a

modified Gompertz equation (2) [33] which has been found to be an appropriate model for describing the progress of cumulative biogas production in batch experiments:

$$H(t) = Ps \exp\left\{-\exp\left[\frac{R'e}{P}(\lambda - t) + 1\right]\right\} \quad (2)$$

where $H(t)$ is the cumulative H_2 production (mL) at fermentation time t (h), P is the potential H_2 production (mL), R' is the maximum H_2 production rate (mL/h), $\ddot{e}$ is the lag phase (h), and e is the exp(1) = 2.71828.

2.2.7 *Analytical methods*

Gas products were analyzed using a gas chromatograph (Focus GC, by Thermo) equipped with a thermal conductivity detector (TCD) and a 3 m Stainless Steel column packed whit Hayesep Q (800/100 mesh). Nitrogen gas was used as a carrier at a flow rate of 35 ml/min. The temperature of the column and of the injector, was 120°C, while that of thermal conductivity detector (TCD) was 200°C.

The metabolic fermentation products (volatile fatty acids, lactate, ethanol and 2,3-butanediol) and glucose consumption were analyzed by a high performance liquid chromatograph (Thermo Spectrasystem P4000) equipped whit both an UV detector (ʎ = 210 nm) and a refractive index detector. The column, a 300 mm × 7.8 mm Rezex ROA-Organic Acid H+ (8%) column (Phenomenex) with a 4 x 30 mm security guard cartridge Carbo-H (Phenomenex), was operated at 65°C, using a solution of 5 mN H_2SO_4 as mobile phase (flow rate, 0.6 ml/min). The liquid samples were diluted 1:10 in 5 mN H_2SO_4 and filtered with 0,22 μm membrane before injection to the HPLC.

2.3 Results and Discussion

2.3.1 *Hydrogen production by self-fermentation of vegetable waste*

The substrates used in this study were common domestic vegetables refuses, coming from the first stage of food processing, and represented a typical waste daily produced throughout the year in the cafeteria at the ENEA Casaccia Research Centre. The total

solid (TS) and the volatile solid (VS) of the substrates, were respectively 5.33% and 4.15% for leaf-shaped refuses (V) and 11.68% and 10.49% for the mix (VP).

Table 1 summarizes the results of batch experiments from wastes. H_2 production from self-fermentation of both substrates was found to be feasible in all the experimental conditions tested. Methane was never detected during the whole fermentation period. The lack of a buffer system produced a rapid decrease of the pH of the culture medium, from pH 7.0 to pH 5.0-4.5, resulting in an inhibitory effect on H_2 production and a shift of the main end-product from acetate to lactate (Figure 1). The temperature affected the H_2 percentage of biogas (Table 1), since, interestingly, the highest concentrations of H_2 (42%) was obtained at temperature closest to ambient value, i.e 28°C. The highest yield of 23.9 ± 2.15 LH_2/ Kg VS was obtained with V substrate at 28°C using phosphate buffer, while is worth to notice that the highest percent of H_2 (32-42%) and the highest amounts of H_2 (184-198 ml) were obtained with buffered VP substrate, characterized by a higher VS content.

Yields were comparable with those previously reported in literature as showed in table 2, which summarizes the maximal H_2 yields in mesophilic batch and continuous self-fermentation, without substrate treatment.

The pH control mode is also reported. In accordance with our results, the pH control of the culture medium proved to be a critical parameter. In Shimizu et al. (2008) [8] the H_2 yield from food waste increased from 40 to 90 ml H_2/gTS when the pH was controlled at 5.0-6.0. In Stamatelatou et al. (2011) [6] the direct pH control (5.2), using NaOH, led to twice H_2 production rate from cheese way (4.8 L/L/d), in comparison with the previous 2.5 L/L/d, attained in a reactor with pH regulate by alkalinity addition [9].

The H_2 production results by self-fermentation process reported in this study and in previous researches (Table 2), demonstrated that the use of indigenous waste microflora was an applicable and desirable practice. From an industrial and commercial perspective, this can facilitate the H_2 fermentation process and reduce energy consumption [10].

2.3.2 *Isolation and selection of H_2-producing bacteria*

A total of sixty-three single distinct colonies, were retrieved and designated by progressive numbers of isolation: 16 were obtained from V (numbered from 1 to 16)

and 47 from VP (numbered from 17 to 63). H_2-producing bacteria were successfully isolated from both V and VP biomass. Among the isolates that grew on liquid medium, eleven (seven V-isolates: 2, 3, 4, 5, 9, 10, 15; and four VP-isolates: 47, 54, 56, 57) were selected for their ability to produce H_2 from glucose under anaerobic conditions, with H_2 yields ≥ 0.2 mol H_2/mol of glucose and a H_2 content in the evolved biogas ranging from 13 to 52% (data not shown).

2.3.3 *Identification and phylogenetic analysis of selected strains*

The 16S rRNA gene sequences of selected isolates have been determined and deposited onto NCBI GenBank under accession number: FJ587222.1 (2); FJ587223.1 (3); FJ587224.1 (4); FJ587225.1 (5); FJ587226.1 (9); FJ587227.1 (10); FJ587228.1 (15); FJ587229.1 (47); HM627386.1 (54); FJ587230.1 (56); FJ587231.1 (57). Comparison with the 16S rRNA genes in the GenBank showed that all the selected isolates had sequence similarities ≥98% with known strains (Table 3), ten belong to the family of *Enterobacteriaceae* (*γ–proteobacteria*) and one to the *Streptococcaceae* (*Firmicutes*). No obligate anaerobes were found among the H_2 producers. From the analysis of 16S rRNA gene sequence by BLASTn, it was found that the eleven isolates could be affiliated with bacteria in 8 different genera which were *Pectobacterium*, *Buttiauxella*, *Enterobacter*, *Rahnella*, *Raoultella*, *Pantoea*, *Citrobacter* and *Lactococcus.* The phylogenetic tree is shown in Figure 2.

Bacteria belonged to these genera are chemoorganotrophic, facultatively anaerobic, having both respiratory and fermentative type of metabolism [34]. The genera *Enterobacter*, *Pantoea*, *Citrobacter* were already known for their H_2 production ability [35-38]. While, as far as the authors are aware, the genera *Pectobacterium*, *Buttiauxella*, *Rahnella and Raoultella* were described as potential H_2 producers for the first time in this study. Bacteria belonging to the genus *Lactobacillus* are often found in microbial fermentative communities, although many Lactobacillus spp could not produce H_2. However, Yang et al. (2007) [39] reported that *Lactobacillus* spp were the predominant microorganisms in the bioreactors when higher H_2 yields were obtained. Accordingly to these authors and to Castello et al. (2009) [40], we suggest that the H_2 production capacity of the genus Lactobacillus should be re-evaluated.

2.3.4 *Effect of initial glucose concentration on H_2 production of selected strains*

Batch tests were carried out, for each isolate, to investigate the effect of glucose concentration (1-5-10 g/l) on fermentative H_2 production at 28 °C and under the initial pH of 6.7. The isolates 3 and 54 showed very low H_2 yield (≤ 0,3 mol H_2/mol glucose) only at glucose concentration of 10g/l and for these reasons they were not considered in further discussions. Table 3 summarized the ranges of H_2 production performance parameters for the other isolates. The results revealed that the hydrogen production yield decreased with an increase in the concentration of the initial glucose from 1 to 10g/l. Higher hydrogen production yields (1.58-2.19 mol H_2/mol glucose) occurred at the initial glucose concentration of 1g/l after 24-27 hours while the maximum volumetric H_2 production was obtained at a initial substrate concentration of 10g/l and ranged from 410 to 1016 ml/l.

It is interesting to point out that, with the exception of isolate 3 (as mentioned before), all isolates affiliates to the genera *Pectobacterium, Buttiauxella, Rahnella and Raoultella,* which had never been previously studied for H_2 production, have proved to have good performances. In particular, isolate 10 and 47 showed the highest yields, 2.10 ± 0.15 and 2.19 ± 0.07 mol H_2/mol glucose respectively, at initial glucose concentration of 1g/l. The obtained yields exceed the theoretical limit of 2 mol H_2/mol glucose reported for facultative anaerobic bacteria [41]. It is possible to hypothesize that the H_2 produced could be derived from source other than glucose, mainly protein hydrolysates of yeast and peptone extract contained in culture medium. Although [35] reported that peptone added in the medium did not affect the production of H_2, by *Enterobacter aerogenes*, [42] reported that approximately 12-15% of the production of biogas, in the case of H_2 production by *Thermotoga neapolitana*, required consumption of protein source. However, both authors highlight the importance of using yeast extract in the culture medium to support the initial phase of bacterial growth. Although the isolates showed different performances at different initial glucose concentrations, the modified Gompertz equation was found to be an appropriate model to fit experimental data. The S-shaped curve for biogas evolution was typical for all performed experiments, confirmed by good R^2 coefficients (> 0.97).

The results also showed that hydrogen partial pressure (pH_2) range, in the headspace of batch culture increased with increasing initial glucose concentration and affected the

fermentative hydrogen production leading to decrease yields. H_2 producing isolates performed a mixed acid fermentation of glucose, forming acetic acid, formic acid, ethanol and lactic acid. When using higher substrate concentrations, a higher lactate level and correspondingly a lower acetate one, were detected (Figure 2). Moreover a positive and significant Spearman' s rank correlation coefficients between highest pH_2 in the headspace of batch cultures of all isolate, and lactate and ethanol concentration at the end of fermentation, were observed (R = 0.72 and 0.64, respectively; N= 27; $p< 0.01$).

These results are in concurrence with earlier reports which revealed that builds up of hydrogen gas in the headspace of batch culture resulted in the decrease of the hydrogen yields [43-46]. In particular, Jungare et al., 2011 [46] reported an increase in hydrogen yield from 2.65 to 3,1 mol of H_2/mol glucose with the decrease in hydrogen partial pressure to 1.01×10^4 Pa. Moreover high pH_2 is known to lead to production of more reduced substrates, such as lactate and ethanol, that drain the electron required for H_2 production. On the other hand van Niel et al., (2003) [47] reported that lactate production started at hydrogen partial pressure higher than 0.98×10^4 Pa.

2.4 Conclusion

The results of this research showed that vegetable waste successfully served not only as a substrate, but also as a source of suitable inocula. Self-fermentation of common domestic vegetable refuses produced H_2 at temperature of 28°C and 37°C without the need of chemical or physical pre-treatment of the substrate. Methane production was never detected. The highest yields (23.9 l/kg of VS) and H2% were obtained at 28°C, only by control of pH.

From an industrial and commercial perspective, the self- fermentation process at temperature close to ambient temperature seems to be advantageous.

Screening microbial diversity of the waste can be a successful strategy to select highly efficient H_2-producing bacteria. 11 bacterial isolates were obtained directly from waste samples, one was identified to be member of the family *Streptococcaceae* and ten of the family *Enterobacteriaceae.* The found genera *Pectobacterium, Buttiauxella, Rahnella*

and Raoultella were characterized as potential H_2 producers for the first time in this study.

The hydrogen production yields and the metabolic pathways of the isolates were affected by substrate concentration and hydrogen partial pressure. The results indicated that higher hydrogen production yields (1.58-2.20 mol H_2/mol glucose) occurred at the initial glucose concentration of 1g/l at $pH_2 \leq 0.98 \times 10^4$ Pa. Furthermore, high pH_2 leaded to metabolic pathways shift towards production and accumulation of more reduced substrates, such as lactate and ethanol.

The high conversion efficiency of the new hydrogen producers, obtained at glucose concentration of 1g/l, make them new candidates for biological hydrogen production at low temperature (28°C).

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2.6 Tables and Figures

Table 1. Hydrogen production by self-fermentation of biomasses V and VP in both presence and absence of 0.1 M phosphate buffer at different temperature. Average values are reported, standard deviation ranged from 8 to 17%.

Biomass	Liquid Culture	T°C	Fermentation Time (h)	H_2	H_2 (ml)	L H_2/ KgVS	pH initial/ final
V	phosphate buffer	28	51	27.96	99.08	23.88	6.8 / 6.0
		37	72	15.76	63.50	13.34	
V	tap water	28	74	10.62	36.59	8.82	7.0 / 4.5
		37	48	0.10	0.33	0,07	
VP	phosphate buffer	28	51	42.00	183.97	17.54	6.8 /6
		37	72	32.00	198.37	19.10	
VP	tap water	28	74	10.23	34.97	3.33	7.0 / 5.0
		37	48	1.00	2.50	0.25	

Table 2. Maximal hydrogen yield and volumetric H_2 production from literature data and from present study on batch and continuous self-fermentation without pretreatments.

Feedstock/Seed	Operation mode	T (°C)	pH controller	L H_2/Kg TS	L H_2/L or Kg biomass	Reference
Cow waste slurry	batch	60	no	22.1	0.39	[11]
Undiluited Cow Dung	batch	60	no	5.05	0.74	[11]
Food waste	batch	35	no	40.0	1.33	[8]
Food waste	batch	35	automatic	90.0	3	[8]
Poultry slaughterhouse sludge	batch	37	no	8.83	n.d.	[12]
Food waste	batch	35	NaOH	4.37	n.d.	[2]
Sewage sludge	batch	37	no	0.25	0.00	[13]
Sweet sorghum extract	continuos	35	NaOH-KH_2PO_4	n.d.	10.0	[14]
Cheese Whey	continuos	35	$NaHCO_3$	n.d.	2.49	[9]
Food waste	semi-continuos	40	no	55.8	n.d.	[10]
Garbage slurry	sequencing batch	37	automatic	31.1	n.d.	[3]
Cheese Whey	continuos	35	automatic	n.d.	4.80	[6]
Leaf shape vegetables waste	batch	28	KH_2PO_4–Na_2HPO_4	18.6	0.99	This study
Leaf shape vegetables waste + potato peels	batch	37	KH_2PO_4–Na_2HPO_4	17.0	1.98	This study

n.d. not determined

1 Table 3 Phylogenetic affiliations of 16S rRNA gene of isolates.

Isolate (Source)	Accession no.	Closest relative [a]	Accession no.[a]	Identity [a]	Band phylogenetic affiliation [b]				
					Phylum	Class	Order	Family	Genus
2 (V)	FJ587222.1	*Pectobacterium carotovorum* strain C150 (type II)	JF926744.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Pectobacterium (100%)
3 (V)	FJ587223.1	*Pectobacterium carotovorum* strain C267	JF926746.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Pectobacterium (100%)
4 (V)	FJ587224.1	*Pantoea* sp. 57917	DQ094146.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Buttiauxella (100%)
5 (V)	FJ587225.1	*Pectobacterium carotovorum* strain C150 (type I)	JF926743.1	0,98	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Pectobacterium (100%)
9 (V)	FJ587226.1	*Enterobacter* sp. 638	CP000653.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Enterobacter (51%)
10 (V)	FJ587227.1	*Rahnella* sp. Y9602	CP002505.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Rahnella (57%)
15 (V)	FJ587228.1	*Enterobacter asburiae* LF7a	CP003026.1	0,98	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Enterobacter (80%)
47 (VP)	FJ587229.1	*Enterobacter* sp. AP11	HM628704.1	1	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Raoultella (96%)
54 (VP)	HM627386.1	*Pantoea* sp. II_Gauze_W_10_17	FJ267564.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Pantoea (100%)
56 (VP)	FJ587230.1	*Lactococcus lactis* strain 15	JF831165.1	0,99	Firmicutes (100%)	"Bacilli" (100%)	"Lactobacillales" (100%)	Streptococcaceae (100%)	Lactococcus (100%)
57 (VP)	FJ587231.1	*Citrobacter freundii* strain JCM 24064	AB548829.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Citrobacter (99%)

(a)Sequence similarities between 16SrDNA gene sequences of isolates and those of the closest relatives in the NCBI database.

(b)Identification performed with RDP Classification Algorithm. Bootstrap confidence values are given between brackets (classification is well supported for confidence > 80%).

Table 4. Ranges of H_2 production parameters of nine selected strains, using glucose at three different concentrations. Average values are reported. Standard deviation ranged from 2.6 to 8%.

GLUCOSE (g/l)	YIELD mol H_2/mol glucose	ml H_2/l	% H_2	pH_2 (Pa x 10^4)
1	1.58-2.19	212-300	14.0-17.3	1.27-1.86
5	0.52-1.10	356-696	21.6-30.0	2.16-4.12
10	0.30-0.74	410-1016	22.3-40.0	2.54-5.49

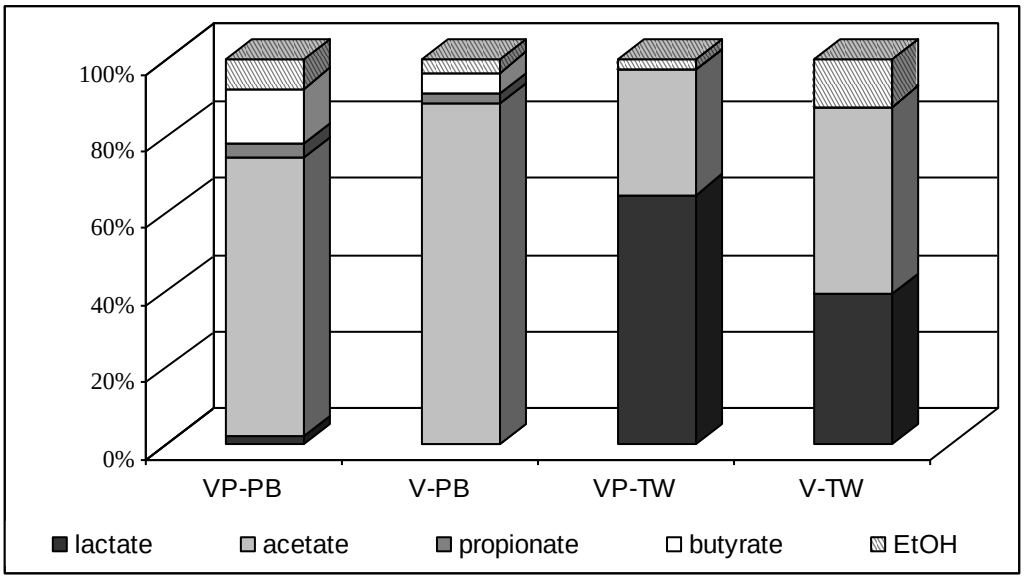

Figure 1. Percentage composition of metabolic products at the end of self-fermentation of V and VP biomasses in phosphate buffer (V-T and VP-T) and in tap water (V-TW e VP-TW) at 37°C. Analysis were repeated twice and average values are reported. The worse case error was within 15%.

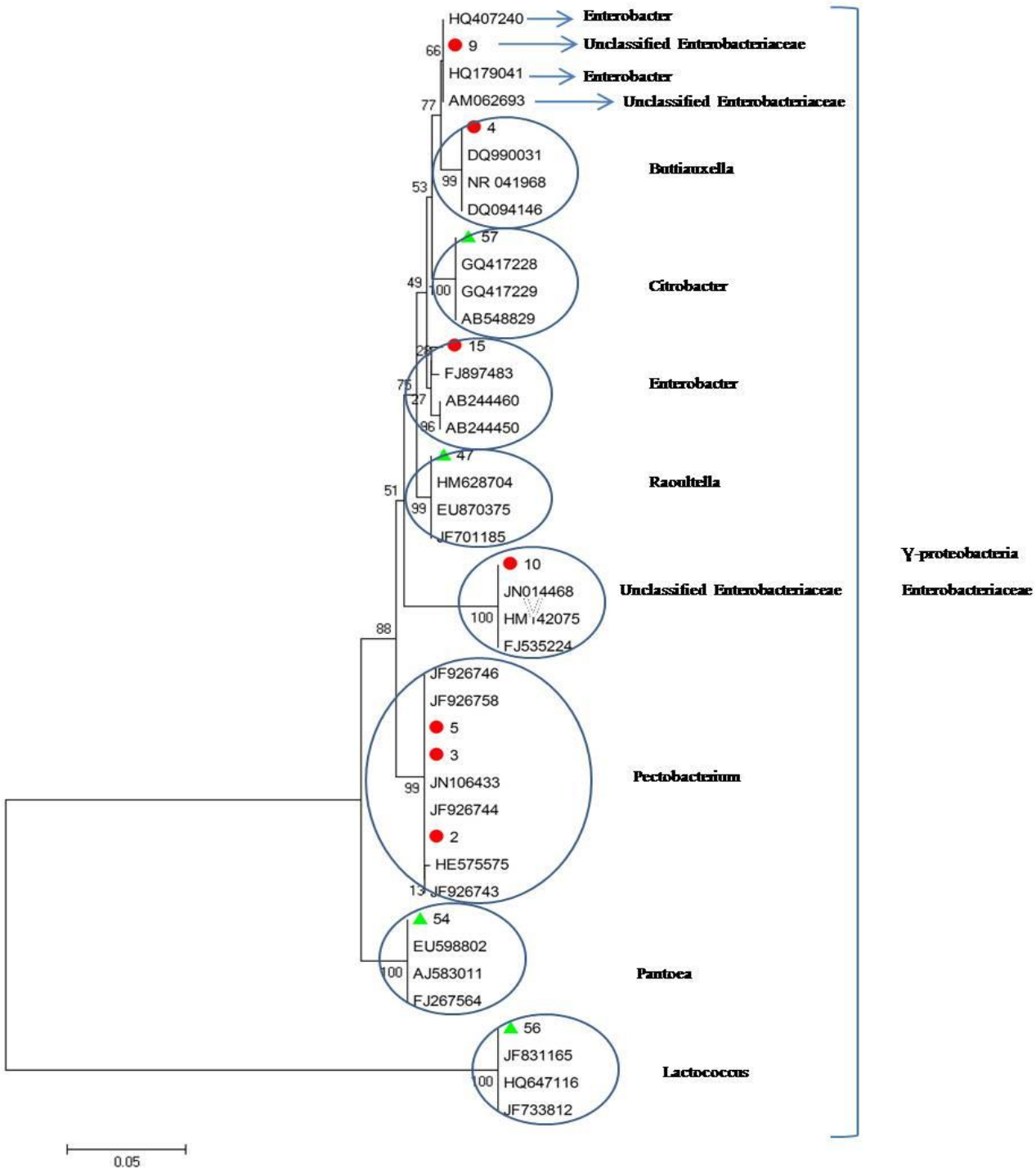

Figure 2. Phylogenetic tree showing the relationship between eubacterial 16SrRNA gene sequences of isolates from V (●) and from VP (▲) and reference sequences obtained through BLAST analysis. Strains out of the circles, with less than 80% confidence, are displayed under an "unclassified" taxon.

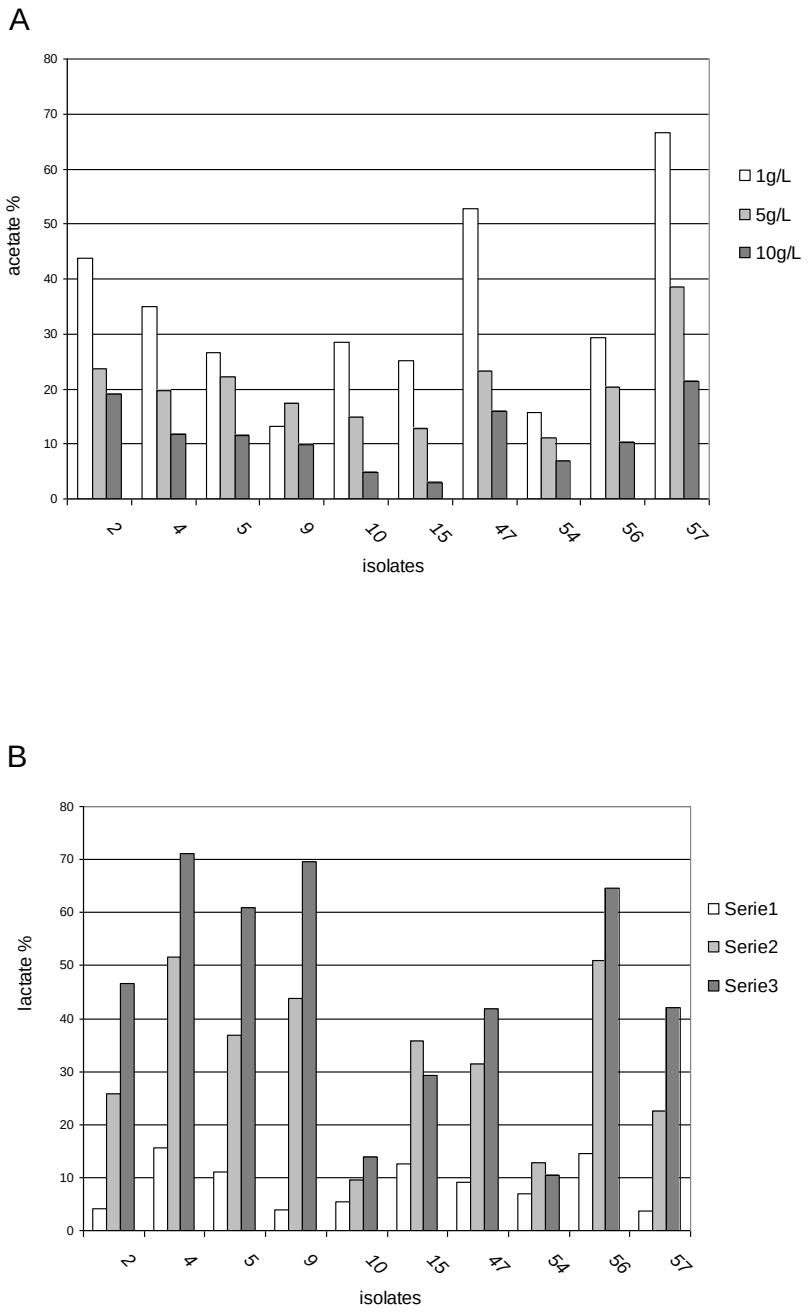

Figure 3. Effect of glucose concentration on acetate (a) and lactate (b) concentrations at the end of fermentation. Reported values were estimated as amount % respect to the total metabolic product detected (VFA, lactate and ethanol). Average values are reported. The worse case error was within 10%.

3. SCREENING MICROBIAL DIVERSITY FROM VEGETAL WASTES IN AID OF BIO-HYDROGEN PRODUCTION

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ABSTRACT

Hydrogen production by dark anaerobic fermentation of organic wastes is a promising strategy to obtain renewable and clean energy in a sustainable way: it solves the problem of energy production and waste disposal at the same time. A pivotal question in bio-hydrogen production processes is the isolation and identification of new highly efficient bio-hydrogen producing anaerobic bacteria.

In this study microbial strains were isolated from non pretreated vegetable kitchen waste, under mesophilic anaerobic conditions. 11 strains out of 63 isolated were selected on the basis of H_2 production capacity. Comparison with the 16S rRNA genes in the GenBank showed that all the producers had sequence similarities ≥ 98% with known strains. Ten were affiliated with phylotype of *Enterobacteriaceae* (γ – *proteobacteria*) and one of *Streptococcaceae* (*Firmicutes*). Seven strains (*Erwinia sp.* MK14; *Pantoea agglomerans* and *P.* sp.57917; E*nterobacter* sp.638 and E. *aerogens* strain C4-1; *Lactococcus lactis* strain R-30; *Citrobacter freundii* strain MH0711) belonged to species already known as hydrogen producers, while four strains (*Pectobacterium carotovorum* strain E161 and strain NZEC23; *Endophyte bacterium* SS10; *Raoultella ornithinolytica*) were characterized for the first time for their ability to produce H_2.

Hydrogen producing performance of isolates was investigated in batch by dark fermentation using glucose as substrate and compared under the same experimental conditions. Cumulative H_2 production, H_2 yield and the amount of soluble metabolites were determined for each strain.

3.1 Introduction

Hydrogen is considered to be the fuel of the future mainly due to its high conversion efficiency, recyclability and non polluting nature. Among the methods for its production, the most promising and environmentally friendly one seems to be the dark fermentation of organic wastes as it solves both the problem of energy production and waste disposal (Benemann, 1996).

The dark H_2 fermentation is carried out by fermentative H_2-producing microorganisms, such as facultative and obligate anaerobes.

One way to improve the efficiency of H_2 production is to identify and to select bacterial strains with high H_2 producing abilities from different substrates (Kalia & Purohit, 2008), and to characterize the microbial metabolism, in order to understand and optimize the whole process.

This work has two main aims: 1) to isolate and to identify potential H_2 producer bacterial strains developed in vegetal waste under mesophilic anaerobic conditions; 2) to characterize the fermentation process of the isolates in batch cultures using glucose as a nutrient source.

3.2 Materials and Methods

3.2.1 *Vegetable kitchen waste*

Vegetable kitchen waste, composed of green vegetables (V) and a mix of green vegetables and potatoes peelings (VP) was chosen as substrate. The waste was collected from the canteen at the ENEA Research Centre-Casaccia.

It comes from the first processing of food and represents a typical daily waste produced during the year.

3.2.2 *Screening for H_2 producing bacteria*

The isolation of microbial colonies was organized as follows: two extracts were obtained by homogenizing 1g of the samples V and VP in sterile saline solution (0.9% v/v). Serial dilutions of the two extracts were plated on the basal fermentation medium (BFM) with 0.1 M KH_2PO_4–Na_2HPO_4 buffer solution, pH 6.8 (Pan et al., 2008). Plates were incubated at 28°C for 48h in anaerobic jar. In order to isolate bacteria, single distinct colonies showing different morphologies were picked up from plates of 10^2-10^3-10^4 fold dilutions. Each colony was re-streaked on fresh plates under anaerobic chamber more than three times to ensure the purity of the isolates. All isolates were cryopreserved in glycerol (30%v/v) at -80°C until use.

Single colonies were picked up from BFM plates and inoculated in 25 ml bottles containing 10 ml of BFM liquid medium and H_2-producing bacteria were selected on the basis of the presence of H_2 in the evolved gas.

3.2.3 *16S rDNA sequencing and phylogenetic analysis*

DNA of each bacterial isolate was prepared by lysis of 2-3 colonies grown overnight on BFM plates according to the procedure described by Vandamme et al., 2002.

The amplification of the 16S rRNA gene was carried out using eubacterial universal primers 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CTACGGCTACCTTGTTACGA-3') with 2µl of each cell lysate suspension in 20µl of Quiagen Taq buffer with 0.5 U of Taq DNA polymerase, as reported by Di Cello et al., 1997. PCR products were purified using the Sephadex TM G-100 resin according to the supplier's instructions and quantitatively analyzed using the spectrometer Thermo Scientific NanoDropTM.

Sequencing reactions were prepared using the sequencing kit Applied Biosystem Big Dye Terminator ® version 3.1, according to the manufacturer's instructions, and analyzed with an ABI PRISM 310 Genetic Analyzer Perkin-Elmer, at the ENEA Genome Research Facility DNA Sequencing Laboratory (Genelab, ENEA C.R. Casaccia, Italy). Thermal cycling was performed with a gene Amp PCR System 9700 instrument (Applied Biosystems).

The raw sequences of both strands were analyzed by the software Chromas Pro and DNA star and the consensus sequences were compared with those deposited using the BLAST network service of the NCBI db (http://www.ncbi.nlm.nih.gov/BLAST/). For phylogenetic analysis, identification of 16S rRNA gene sequences was performed with RDP Classification Algorithm (http://rdp.cme.msu.edu/classifier/classifier.jsp).

3.2.4 *Batch tests for H_2 production*

The bacterial strains were renewed in pre-culture basal medium as reported in Pan et al., 2008. After anaerobic incubation at 28°C with shaking at 120 rpm for 24h, the cultures were used as inocula. The production tests for each strain were carried out in 125 ml serum bottles, containing 50 ml of BFM. The medium was sterilized by autoclaving (121°C for 15 min) while the minerals, vitamins and glucose, previously prepared in stock and filtered with filters 0,22 µm, were added later. The bottles capped with rubber stopper were inoculated 1% (v/v) ratio, with pre-activated bacterial cells and flushed for 2 min with filtered (0.22µm) pure nitrogen gas to remove oxygen. The bottles were incubated in the dark at 28°C in an orbital shaker at 120 rpm. The total gas volume

was measured using water displacement equipment (Kalia et al., 1994). The pH of the cultures was also measured, while liquid and gaseous product composition was analyzed, as described below.

3.2.5 *Analytical methods*

Biogas analysis

The H_2 percentage of biogas in the headspace of the reactors was measured using a gas chromatograph (Focus GC, by Thermo) equipped with a thermal conductivity detector (TCD) and a 3 m Stainless Steel column packed whit Hayesep Q (800/100 mesh).

The volume of produced H_2 was calculated by the mass balance equation (Logan et al., 2002):

$$V_{H,i}=V_{H,i-1}+C_{H,i}(V_{G,i}-V_{G,i-1})+V_H(C_{H,i}-C_{H,i-1})$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative H_2 gas volumes at the current (i) and previous (i–1) time intervals, respectively; $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at the current (i) and previous (i–1) time intervals; $C_{H,i}$ and $C_{H,i-1}$ are the fraction of H_2 gas in the headspace at the current (i) and previous (i–1) time intervals, and V_H is the total volume of headspace in the reactor.

Fermentation products

The metabolic products of fermentation (Volatile Fatty Acids, lactate, and ethanol) and glucose were analyzed by a high performance liquid chromatograph (Thermo Spectrasystem P4000) equipped whit both an UV detector (λ= 210 nm) and a refractive index detector.

The column, a 300 mm × 7.8 mm Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex) with a 4 x 30 mm security guard cartridge Carbo-H (Phenomenex), was operated at 65°C, using a solution of 5 mN H_2SO_4 as mobile phase (flow rate, 0.5 ml/min). The liquid samples were diluted 1:10 and pretreated with 0,22 μm membrane filter before injection to the HPLC.

3.3 Results

3.3.1 *Diversity of microbial isolates*

Totally 63 single distinct colonies were selected, showing different morphologies, 16 from V (numbered from 1 to 16) and 47 from VP (numbered from 17 to 63). 11 of the isolates that were grown on liquid medium, were selected for H_2 production (yield ≥ 0.2 mol H_2/mol glucose): seven V-isolates (2, 3, 4, 5, 9, 10, 15) and four VP-isolates (47, 54, 56, 57). Comparison with the 16S rRNA genes in the GenBank showed that all isolates had sequence similarities ≥98% with known

strains (table I). Ten isolates were affiliated with phylotype of *Enterobacteriaceae* (γ–*proteobacteria*) and one of *Streptococcaceae* (*Firmicutes*).

3.3.2 *H_2 production by microbial isolates*

Preliminary tests were carried out for evaluating the effect of glucose concentrations (5.5 mM, 27.5 mM, 55.5 mM) on H_2 yield (mol H_2/mol glucose). Results show (figure 1) that the yield decreases with increasing substrate concentrations for all strains except for strains 3 and 54. The higher efficiency was observed at glucose 5.5 mM for all the strains except for 3 and 54 (55.5 mM). These concentrations were used for production tests.

Results of H_2 production expressed by all strains are summarized in table II. The gases detected from the anaerobic fermentation process were H_2 and CO_2; CH_4 was never detected. Nine strains (2-*Pectobacterium carotovorum* E161, 4-*Pantoea* sp.57917, 5-*Pectobacterium carotovorum* strain NZEC23, 9-*Enterobacter* sp.638, 10-*Endophyte bacterium* SS10, 15-*Enterobacter aerogens* C4-1, 47-*Raoultella ornithinolytica*, 56-*Lactococcus lactis* R-30 and 57-*Citrobacter freundii* MH0711) were able to utilize glucose for H_2 production and they revealed as good H_2 producers. In contrast, two strains (3-*Erwinia* sp.MK14 and 54-*Pantoea agglomerans*) could not utilize glucose and no H_2 production was observed. The H_2 production yield showed a range from 1.6±0.0 to 2.7±0.2 (mol H_2 /mol glucose). *Pantoea* sp.57917 exhibited the best Hydrogen producing performance corresponding to cumulative production of 18.3±1.5 ml and 19%.

H_2 production followed a variable trend during the experiments: an earlier production (3-6 hours) was observed for *P. carotovorum* E161, *Enterobacter* sp.638, *E.bacterium* SS10, *R. ornithinolytica* and *C. freundii* and a more prolonged time of production was expressed by *R. ornithinolytica* that ended production after 48 hours.

pH of 6.8 was found to be suitable for hydrogen production and did not show any significant variation with a slightly decrease to 5.4 for *Enterobacter* sp.638.

3.3.3 *Composition of soluble metabolites*

Soluble metabolites analyses were performed. In table III only results obtained at ended production are summarized. The main metabolites produced from all H_2 positive strains were acetic acid, formic acid and ethanol, while a smaller amount of propionic acid and lactic acid was also formed. A different pattern was observed for *Erwinia* sp.MK14 and *P. agglomerans* strains that produced respectively the higher concentration of lactic acid, formic acid and ethanol. Butyric acid was produced only by *Erwinia* sp.MK14.

In table III residual glucose concentration is also reported. All glucose added was used by all nine producing strains, while residual concentrations were pointed out for not producing strains.

3.4 Discussion

After verifying the capacity of the waste's endogenous microorganisms to produce H_2 in auto-fermentation (Marone, 2009), we isolated eleven bacterial strains from endogenous microbial population of vegetable waste. Among the producing strains *P. carotovorum* strains E161 and NZEC23, *E. bacterium* SS10 and *R. ornithinolytica* were characterized for the first time. Their potential in H_2 production was comparable to the other known strains (Kalia & Purohit, 2008).

The experimental conditions used were suitable for H_2 production from all microbial isolates except for *Erwinia* sp MK14 and *P. agglomerans.* These strains were not able to convert glucose to H_2 as shown from all parameters (tables II and III).

The H_2 producing strains metabolized all glucose forming acetic acid, formic acid and ethanol as soluble metabolites (table III). They expressed a mixed acid fermentation of glucose characterized by two pathways: the acetic and the formic acids fermentations. These are characteristic pathways of *Enterobacteriaceae* anaerobic fermentation that occurs through the action of both periplasmic hydrogenases and of cytoplasmic formate hydrogen-lyase complex (FLH) (Mathews & Wang, 2009). Induction of FLH complex and therefore the degradation of formic acid to H_2 buffers the drop of pH (Hallenbeck, 2009) and this is consistent with our data. Since H_2 is derived from formic acid, and a maximum of two molecules of formates are formed per glucose, maximum H_2 yield can be predicted to be $2H_2$/glucose.

The results of this work show that it could be possible to improve both H_2 production and yield from fermentation of vegetal waste using autochthonous pure bacterial strains as starters.

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3.6 Tables and Figures

Table I - Phylogenetic affiliations of 16S rRNA gene.

Summary of bacterial 16s rDNA sequenced clones								
Strain	**Closest relative** [a]	**Accession no.**	**Identity**	**Band phylogenetic affiliation** [b]				
		(a)	**(a)**	**Phylum**	**Class**	**Order**	**Family**	**Genus**
2 (V)	*Pectobacterium carotovorum* strain E161	AF373189.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Pectobacterium (100%)
3 (V)	*Erwinia* sp. MK14	AY690721.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Pectobacterium (100%)
4 (V)	*Pantoea* sp. 57917	DQ094146.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Buttiauxella (100%)
5 (V)	*Pectobacterium carotovorum* strain NZEC23	EF530547.1	0,98	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Pectobacterium (100%)
9 (V)	*Enterobacter* sp. 638	AM062693.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Enterobacter (49%)
10 (V)	*Endophyte bacterium* SS10	AY842148.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Rahnella (76%)
15 (V)	*Enterobacter aerogenes* strain C4-1	AB244460.1	0,98	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Enterobacter (64%)
47 (VP)	*Raoultella ornithinolytica*	AB004756.2	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Raoultella (98%)
54 (VP)	*Pantoea agglomerans*	EU598802.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Erwinia (93%)
56 (VP)	*Lactococcus lactis* strain R-30	EU483103.1	0,99	Firmicutes (100%)	"Bacilli" (100%)	"Lactobacillales" (100%)	Streptococcaceae (100%)	Lactococcus (100%)
57 (VP)	*Citrobacter freundii* strain MH0711	EU360794.1	0,99	Proteobacteria (100%)	γ – proteobacteria (100%)	Enterobacteriales (100%)	Enterobacteriaceae (100%)	Citrobacter (99%)

a: sequence similarities between rDNA gene sequences of strain and those of the closest relatives in the NCBI database.

b: identification performed with RDP Classification Algorithm. Bootstrap confidence values are given between brackets (classification is well supported for confidence >80%).

Table II - Summary of parameters characterizing H_2 production by all isolates.

MICROBIAL ISOLATES		time of production [c]		production efficiency[d]			final[e]
		Start (h)	End (h)	H_2 (%)	H_2 (ml)	mol H_2 / mol glu	pH
2 (V)[a]	*Pectobacterium carotovorum* strain E161	6	24	15	17,7± 1,3	2,6± 0,1	6,8
3 (V)[a]	*Erwinia* sp. MK14	17	28	2	2,1± 0,0	0,0	5,2
4 (V)[a]	*Pantoea* sp. 57917	3	24	19	18,3± 1,5	2,7± 0,21	6,6
5 (V)[a]	*Pectobacterium carotovorum* strain NZEC23	9	41	16	14,3± 0,6	2,1± 0,0	6,7
9 (V)[a]	*Enterobacter* sp. 638	6	46	14	11,3± 0,2	1,6± 0,0	5,4
10 (V)[a]	*Endophyte bacterium* SS10	6	39	16	13,1± 0,2	1,9± 0,0	6,4
15 (V)[a]	*Enterobacter aerogenes* strain C4-1	18	48	14	11,8± 0,6	1,7± 0,1	6,7
47 (VP)[b]	*Raoultella ornithinolytica*	4	48	14	11,8± 2,1	1,7± 0,3	6,4
54 (VP)[b]	*Pantoea agglomerans*	14	21	14	13,3±1,1	0,2± 0,0	6,2
56 (VP)[b]	*Lactococcus lactis* strain R-30	14	48	16	14,5± 0,6	2,1± 0,1	6,7
57 (VP)[b]	*Citrobacter freundii* strain MH0711	6	39	17	14,4± 0,8	2,1± 0,1	6,4

a: V, strains isolated from green vegetables

b: VP, strains isolated from green vegetables and potatoes peelings

c: start time and end time (hours) of H_2 production

d: highest parameters of production

e: medium pH at ended production

Table III - End fermentation soluble metabolites and residual glucose.

MICROBIAL ISOLATES		soluble metabolites and residual glucose (Mm) at ended production						
		Acetic acid	Formic acid	Proprionic acid	Butyric acid	Lactic acid	Ethanol	glucose
2 (V)[a]	*Pectobacterium carotovorum* strain E161	6,9± 0,3	2,6± 0,1	2,2± 0,1	0,0	1,2± 0,0	5,5± 0,1	0,0 *
3 (V)[a]	*Erwinia* sp. MK14	3,6± 0,0	10,8± 0,1	3,1± 0,1	4,2± 0,0	76,7± 0,0	13,1± 0,3	10,7 +
4 (V)[a]	*Pantoea* sp. 57917	7,1± 0,9	3,1± 0,3	1,2± 0,1	0,2± 0,2	1,6± 0,0	5,9± 1,1	0,0 *
5 (V)[a]	*Pectobacterium carotovorum* strain NZEC23	3,9± 0,0	4,7± 0,0	0,0	0,0	3,6± 0,0	3,4± 1,9	0,0 *
9 (V)[a]	*Enterobacter* sp. 638	9,42± 2,3	2,43± 1,6	0,0	0,0	3,61± 1,3	6,12± 1,4	0,0 *
10 (V)[a]	*Endophyte bacterium* SS10	12,5± 0,1	3,35± 1,5	0,0	0,0	1,2± 0,1	11,4± 1,2	0,0 *
15 (V)[a]	*Enterobacter aerogenes* strain C4-1	5,9± 1,5	4,7± 0,3	2,2± 0,7	0,0	1,9± 1,2	3,8± 1,5	0,0 *
47 (VP)[b]	*Raoultella ornithinolytica*	10,4± 3,6	2,5± 0,3	0,0	0,0	2,2± 0,5	9,5± 1,3	0,0 *
54 (VP)[b]	*Pantoea agglomerans*	0,0	19,1± 0,0	0,0	0,0	11,3± 0,0	24,7± 0,2	2,7 +
56 (VP)[b]	*Lactococcus lactis* strain R-30	7,3± 0,7	3,9± 0,1	4,4± 0,2	0,8± 0,1	3,3± 0,1	4,2± 0,3	0,0 *
57 (VP)[b]	*Citrobacter freundii* strain MH0711	6,9± 2,1	1,3± 0,2	0,0	0,0	2,2± 0,5	4,9± 1,3	0,0 *

a: V, strains isolated from green vegetables

b: VP, strains isolated from green vegetables and potatoes peelings

*: 5.5mM of glucose added

+: 55.5mM of glucose added

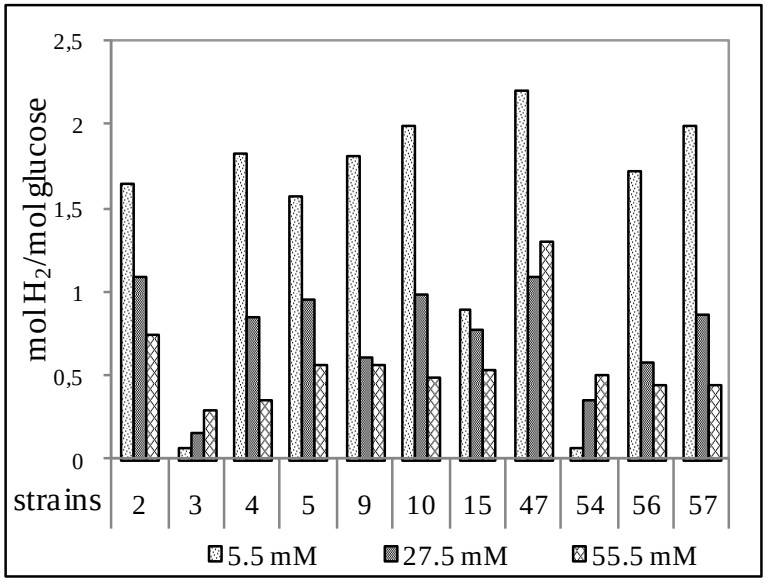

Figure 1 - Effect of glucose concentrations on H_2 production efficiency.

4. HYDROGEN PRODUCTION FROM VEGETABLE WASTE BY BIOAUGMENTATION OF INDIGENOUS FERMENTATIVE COMMUNITIES

ANTONELLA MARONE*, GIULIA MASSINI, CHIARA PATRIARCA, ANTONELLA SIGNORINI, CRISTIANO VARRONE AND GIULIO IZZO

ABSTRACT

This work adopted an innovative approach for fermentative H_2 production from common domestic organic waste, at 28°C, in the absence of pre-treatment: the self-fermentation of non-sterile vegetable waste and the bioaugmentation of microbial indigenous fermenting communities. For this purpose, three new H_2-producing strains, *Buttiauxella* sp. 4, *Rahnella* sp. 10 and *Raoultella* sp. 47, isolated and enriched from vegetable waste, were individually tested on two types of vegetable waste and compared with a bacterial artificial consortium composed of the three strains put together. The three single strains were also characterized for their ability to produce H_2 on different sugars, such as xylose, arabinose and cellobiose, as these are key products of hydrolysis of cellulose and hemicelluloses. H_2 production occurred from self-fermentation with values ranging from 18.08 and 21.95 ml H_2/g VS. All bacterial inocula promoted a significant increase of the H_2 yield and the H_2 production rate, compared to the self-fermentation. The inocula of the artificial consortium yielded the maximum H_2 production of 85.65 ml H_2/g VS with the highest H_2 production rate of 2.56 ml H_2/h.

Keywords: Self-fermentation; Bioaugmentation; Fermentative H_2 production; Facultative anaerobes; Cellulose and hemicellulose hydrolyzate.

4. 1 Introduction

The green economy, based on the ecological economics approach [1] makes it necessary to focus research efforts on renewable energy sources. Discarded biomass residues from forestry, agriculture and municipal waste are potential bioenergy sources, which are not well managed at present and thus pose significant environmental problems [2]. Anaerobic dark fermentative process attracts increasing interest among the technologies useful to extract energy from biomass as it produces H_2 and other high-value products [3-7]. Hydrogen is an important energy vector as its combustion produces only water vapor, it has an energy yield 2.75 times higher than hydrocarbons [8], and it can be easily converted into electricity by fuel cells [9,10]. In Italy, waste biomass production reaches about 43.31 Mt dry weight/year with a potential H_2 production of about 2.84 Mt [11]. In accordance with our previsions, the energy recovery can be estimated in almost 40 TWh / year, that is more than 10% of the net national electricity demand.

Studies on H_2 production by dark fermentation are mainly carried out using a single carbon source or hydrolysis products of lignocellulosic biomass (glucose, sucrose, cellobiose, starch hydrolysate) as model substrates [12]. Although in these findings relatively high H_2 yields were reported, they are not reflective of productivity from actual organic waste. Moreover pretreatments of inocula and/or substrates are widely used to suppress H_2 consuming bacteria [13], to enrich H_2 producing community [14] and to promote hydrolysis of complex substrates [15]. Heat pretreatment of seed inocula is the most commonly used procedure to suppress the H_2-consuming bacteria (methanogens) and to select spore forming, H_2-producers. However it was shown to be weak or totally inefficient with H_2-consuming omoacetogens elimination [16], while, suppress non-spore-formers, H_2-producing, facultatives, such as those belonging to the family of Enterobacteriaceae.

This loss of diversity can lead to a drop in functionality, which translates into reduction of efficiency of the whole process. To maximize the production of H_2 from complex substrates, such as vegetable waste, it is desirable to have a high number of metabolic pathways that promote the hydrolysis of biomass and the metabolism of hydrolysis products. Furthermore the use of obligate anaerobes, such as *Clostridium* species, requires efficient active control and maintenance of strictly anaerobic atmosphere. This makes the use of facultatives in dark fermentative processes more advantageous than strict anaerobes, since the former are less sensitive to O_2 [17].

A possible alternative to expensive and energy consuming pretreatment could be the bioaugmentation, i.e. the practice of adding specific microorganisms to a substrate for the improvement of hydrogen production, regardless of whether the substrate is subjected to chemical and physical treatments.

Recent studies demonstrate the effectiveness of bioaugmentation to implement fermentative H_2 processes. Wang et al. (2008) [18] 8.1 mmol H_2 g^{-1} cellulose using a co-culture of *C. acetobutylicum* with strain *E. harbinense*, the highest amongst those reported in literature to produce H_2 from cellulose materials. In 2009, Kotay & Das (2009) [19] explored seven different pretreatment methods to maximize the H_2 yield from sewage sludge. Pretreatment was essential to reduce competitive microbial load and to improve the nutrient solubilisation of sludge, but the pretreatments alone were insufficient in developing a suitable microbial consortium for H_2 production. While, inoculation with a defined microbial consortium of E. cloacae IIT-BT 08, C. freundii IIT-BT L139 and B. coagulans IIT-BT S1 was found to improve H_2 yield by 1.5–4 times with respect to self-fermentation of pretreated sewage sludge. Also, several authors [20-22] reported that specific axenic bacteria can be cultivated as a better alternative to microbial consortia obtained from thermal pretreated sludge, soils or compost, because of the possibility to follow and drive the metabolic pathways and products.

The principal cost of applying this practice to untreated substrate is due to the substrate requirement of producing the axenic enrichments, while production and distribution of specific enriched bioaugmentation cultures, would be time consuming. Even if an untreated substrate undergoes rapid deterioration and requires careful management, while, chemical and/or physical pretreatments, make the substrates more stable by avoiding the rapid deterioration and allowing the storage. However, to scale-up the industrial process for the biological production of H_2 it would be advantageous if such energy-consuming processes could be avoided without detriment to the process.

On the other hand the waste biomass contains itself indigenous microflora including useful strains like H_2-producing and cellulose-degrading bacteria. However, at present only few studies reported biohydrogen production from self-fermentation of solid waste [23-27].

Thus this study explore the feasibility of H_2 production by anaerobic self-fermentation of two kinds of vegetable kitchen waste: leaf-shaped Vegetable refuse (V) and leaf-shaped Vegetable refuse plus Potato peels (VP), without pretreatment and added nutrient. Moreover, to improve the H_2 production by self-fermentation, bioaugmentation of the indigenous microbial community was performed. To this purpose pure culture and bacterial consortium of three different new bacterial strains (*Buttiauxella* sp.4, *Rahnella* sp.10 and *Raoultella* sp.47) isolated and enriched from the same vegetable waste, were inoculated. The effects of the bioaugmentation in terms of H_2 production were investigated, metabolites composition and hydrolytic products consumption were also analyzed.

The three single strains were also characterized for their ability to produce H_2 on different sugars: xylose, arabinose and cellobiose as these are the key products of hydrolysis of cellulose and hemicelluloses. Studies and optimization process of biological production of H_2 have been so far mainly done by using glucose as substrate. However, the ability of microorganisms to use hexoses (glucose, galactose and mannose), pentoses (xylose and arabinose), and several disaccharides (cellobiose and xylobiose) obtained by the hydrolysis of the vegetal biomass, can significantly increase the energy efficiency of the process.

In addition fermentative H_2 production from xylose, arabinose and cellobiose are mainly reported using anaerobic *Clostridium* and sludge which are dominated by *Clostridium* species [28,29]. To our knowledge, only few studies have focused on fermentative H_2 production from pentose sugars [30-32].

4. 2 Materials and Methods

4.2.1 *Microorganism and culture condition*

The strains, used in the present study, were previously isolated from non pretreated vegetable kitchen waste of the same origin as that used in this study: *Buttiauxella* sp.4 (Accession n° FJ587224.1) and *Rahnella* sp.10 (Accession n° FJ587227.1) were obtained from V while *Raoultella* sp.47 (Accession n° FJ587229.1) was isolated from VP [27]. These strains belong to the family of Enterobacteriaceae which are known for their H_2 production ability. In earlier studies, the three strains were investigated and established as potential H_2 producers for the first time. Bacteria belonging to these three genera are widely known as chemoorganotrophic, facultatively anaerobic, having both respiratory and fermentative type of metabolism, metabolically versatile, being able to grow on minimum mineral salts and to utilize a wide range of carbon sources [33]. *Buttiauxella* sp. 4, *Rahnella* sp. 10 and *Raoultella* sp. 47 were reactivated in pre-culture basal medium with the following composition (g/l): glucose 20; peptone 4; NaCl 3; KH_2PO4 1; K_2HPO_4 1; L-Cysteine · HCl 7 · H_2O 0.5; $FeSO_4 \cdot 7H_2O$ 0.1; $MgCl_2$ 0.1; as reported in Pan et al. [34]. After anaerobic incubation at 28°C with shaking at 120 rpm for 24h, the cultures were used as inocula at uniform cell concentration (OD_{600}). A microbial consortium of three strains was constructed at a cell density ratio of 1:1:1, corresponding to a cell concentration of approximately 10^8 cells/ml for each strain.

4.2.2 *H_2 production on vegetable waste in batch experiments*

The vegetable kitchen waste was collected from the cafeteria at the ENEA-Casaccia Research Centre. Two types of waste, produced daily in the first stage of food processing, were utilized: leaf-

shaped vegetables (V) and a mixture composed by 80% wet mass of leaf-shaped vegetables and 20% of potato peels (VP). The mixture composition reflected the average weekly production of these wastes in the cafeteria. No thermal or chemical pretreatments of the waste were performed. The total solid (TS) and the volatile solid (VS) of the waste used in the test, estimated according to standard methods [35], were respectively 5.76% and 4.71% for V and 8.39% and 7.28% for VP. The waste was crushed to less than 5 mm particle size and diluted with phosphate buffer 0.1 M (KH_2PO_4–Na_2HPO_4), pH 6.70, for a final 0.4 w/v ratio.

Hydrogen production experiments were carried out in 125 ml serum bottles with a working volume of 25 ml.

After inoculating 10% v/v (not in the case of self-fermentation tests), the bottles were capped with rubber stopper and aluminum seals, flushed with nitrogen gas to establish anaerobic condition and incubated in a thermostat at 28 °C with orbital shaking of 120 rpm.

During fermentation the total amount of biogas produced (ml), biogas composition (%), the pH and the composition of metabolic (volatile fatty acids, lactic acid and ethanol) and hydrolytic products were analyzed.

4.2.3 *H_2 production on different carbon sources in batch experiments*

Batch dark fermentation experiments were carried out in125ml anaerobic serum bottles with 50 ml of. Fermentation Medium (BFM), slightly modified from that used by Pan et al. (2008) [34], consisting of (g/l): peptone 3; yeast extract 1; L-cysteine · HCl · H_2O 0.5; $FeSO_4$ · $7H_2O$ 0.1; $MgCl_2$ 0.1; 10ml mineral salt solution (contained 0.01g/l $MnSO_4$ · $7H_2O$; 0.05g/l $ZnSO_4$ · $7H_2O$; 0.01g/l H_3BO_3; 0.01g/l $CaCl_2$ · $2H_2O$; 0.01g/l Na_2MoO_4; 0.2g/l $CoCl_2$ · $6H_2O$; 0.01g/l $AlK(SO_4)_2$;· $12H_2O$ 0.001g/l NICl · $6H_2O$) and 5ml vitamin solution (contained 0.01g/l cobalamin, 0.025g/l vitamin C; 0.025g/l riboflavin; 0.02g/l citric acid; 0.05g/l pyridoxal; 0.01g/l folic acid; 0.025g/l creatine) and were dissolved in 1l of KH_2PO_4-Na_2HPO_4 buffer (final concentration 0.1M pH 6.70). After sterilization (121°C for 15 min) mineral salts, vitamins and carbon sources (xylose, arabinose and cellobiose), previously sterilized by filtering with 0.22 µm filter, were added. The bottles capped with rubber stopper and aluminum seals were inoculated 1% (v/v) ratio and flushed with filtered (0.22 µm) pure nitrogen gas, to remove oxygen. The bottles were incubated at 28°C in a thermostat, with orbital shaking of 120 rpm. All the batch tests were carried out independently in triplicates.

Previously, *Buttiauxella* sp. 4, *Rahnella* sp. 10 and *Raoultella* sp. 47 have been investigated for the effect of initial glucose concentration (1, 5 and 10 g/l) on hydrogen production. The results showed that increasing glucose concentration decreased the conversion efficiency (mol H_2/mol glucose) and

a concomitant build-up of lactic acid occurred. Nevertheless a higher concentration of hydrogen in biogas was obtained at intermediate concentration of glucose (5 g/l). These results prompted us to carry out the experiments on xylose, arabinose and cellobiose at the concentration of 5 g/l.
Further, to evaluate the contribution of yeast extract and peptone contained in BFM medium on hydrogen production, the three strains were cultured in BFM without sugar addition.
During fermentation the total amount of biogas produced (ml), biogas composition (%) and the composition of metabolic products (VFA and Ethanol) were monitored.

4.2.4 *Analytical methods*

Biogas analysis

The total biogas volume was measured using a water displacement system [36].
The biogas composition in the headspace of the reactors was analyzed using a gas chromatograph (Focus GC, by Thermo) equipped with a thermal conductivity detector (TCD) and a 3 m Stainless Steel column packed whit Hayesep Q (800/100 mesh). Nitrogen gas was used as a carrier at a flow rate of 35 ml/min. The temperature of the column and of the injector, was 120°C, while that of thermal conductivity detector (TCD) was 200°C. The volume of produced H_2 was calculated by the mass balance equation [37].

Metabolites and hydrolysis products

The metabolic products of fermentation (volatile fatty acids, lactate, and ethanol) and sugars released by hydrolysis of vegetable waste (glucose, cellobiose, xylose and arabinose) were analyzed by a high performance liquid chromatograph (Thermo Spectrasystem P4000) equipped with both an UV detector (λ= 210 nm) and a refractive index detector.
The column, a 300 mm × 7.8 mm Rezex ROA-Organic Acid H^+ (8%) (Phenomenex) with a 4 x 30 mm security guard cartridge Carbo-H (Phenomenex), was operated at 65°C, using a solution of 5 mN H_2SO_4 as mobile phase (flow rate, 0.5 ml/min). The liquid samples were diluted 1:10 in H_2SO_4 5 mN and filtered with 0.22 µm membrane before injection to the HPLC.

Kinetic analysis

The cumulative H_2 production (H) data were fitted to a modified Gompertz equation [38], which has been found to be an appropriate model for describing the progress of cumulative H_2 production in batch experiments. A second modified Gompertz equation [26] was used for the cumulative H_2 production of *Rahnella* sp.10 on arabinose, because it showed the appearance of a second lag-phase. Parameters (Ps, R' and λ) were determined by fitting the cumulative hydrogen production curves,

minimizing the residual sum of square (RSS) between the experimental data and the expected values, using "Solver" function in Microsoft Excel version 12 (Microsoft, Inc., USA).

4. 3 Results and Discussion

4.3.1 *H_2 production on vegetable waste*

The H_2 production performances of all batch tests are shown in Table 1 and Figure 1.

Fermentative H_2 production of non-pretreated vegetable kitchen waste was found to be feasible, also without inoculum addition.

Indeed, the results showed (Figure 1) that H_2 production occurred from self-fermentation of both biomasses V and VP (21.95 ± 5.35 and 18.08 ± 0.66 ml H_2/ gVS respectively). Methane was never detected during the whole fermentation period.

All the single bacterial inocula promoted a significant increase (up to 4 times) of the H_2 yield. (Figure 1).

The H_2 production (ml) was higher for VP substrate, while yields were higher for V substrate, both in the presence and absence of different inocula. Among the single bacterial strains, *Rahnella* sp. 10 with 47.54 ml H_2/ gVS on V substrate and 37.20 ml H_2/ gVS on VP, showed the lowest performance in H_2 production yields. Instead *Buttiauxella* sp. 4 and *Raoultella* sp. 47 showed similar achievement on V substrate, respectively 71.27 ml H_2/ gVS and 69.70 ml H_2/ gVS, while on the substrate VP *Buttiauxella* sp. 4 showed the best performance of 62.45 H_2/ gVS, while *Raoultella* sp. 47 yielded only 47.17 H_2/ gVS. The artificial consortium inoculum yielded the maximum H_2 production (85.65 ± 0.37 and 66.69 ± 3.25 mL H_2/gVS respectively for V and VP). The values were among the highest compared to those previously reported for H_2 fermentation of organic waste, which needed, in most cases, pretreatment of inoculum or substrate for stable performance (Tab 2).

Table 1 summarize the Gompertz equation coefficients and the Pearson correlation between observed and predicted data. The ''S'' shape curve for biogas evolution was typical for all performed experiments indicating excellent R^2 coefficients (> 0.99). Major differences, for both substrates, were observed in the maximum rate of H_2 formation (R_m): the use of individual bacterial strains, on average, doubled the production speed compared to the self-fermentation. The consortium caused the greatest increase, since the rate of H_2 production, compared to the self-fermentation, increased by 3 times, for the substrate V, and more than 4 times for the VP substrate.

At the end of the fermentation, the pH of each batch experiment decreased, from initial 6.7, to a final pH ranging between 5.5 (*Buttiauxella* sp. 4 on V substrate) and 6.4 (for self-fermentation of

VP substrate), due to the accumulation of various organic acids produced by microbial metabolism [48].

4.3.2 *Hydrolytic products from vegetable waste fermentation*

The phase of hydrolysis plays a crucial role in the fermentation of lignocellulosic biomass. It is necessary to employ a hydrolytic microflora which has a high ability to use the different molecules released at this stage. The composition of cellulosic hydrolysates has close connection with the usability of the hydrolyzed substrates in energy producing fermentation [49]. In order to understand the dynamics of the fermentation process and to analyze the efficiency of substrate degradation, the concentration of reducing sugars released after the bacterial hydrolysis and saccharification of cellulosic biomass were determined, during the course of the experiments.

Figure 2a shows typical kinetic profiles of the main sugars (glucose, xylose, arabinose and cellobiose) released by hydrolysis of vegetable waste for one specific example (consortium on VP); trends of H_2 production yield (ml H_2/g VS) are also shown. These kinetics and trends were similar for all performed experiments. In the early stages of fermentation a rapid depletion of glucose and xylose, correlated to the H_2 production, was observed (Figure 2a). The arabinose was slowly released in the solution by hydrolysis of the substrates, and was consumed in the fermentation process only after 20h of incubation. The fermentation of arabinose requires various enzymes, and hence its biochemical reactions are relatively complex [29]. Cellobiose was detected only in small quantities. It could indicate the absence of cellulose hydrolytic enzymes in the fermenting microflora or more likely that the cellobiose obtained through the hydrolysis of cellulose was rapidly hydrolyzed to glucose. In all the experiments, at the end of fermentation, the sugars were completely consumed. *Buttiauxella* sp. 4 was the strain that showed the best cellulolytic performance. In fact at the beginning of the fermentation the glucose concentration was 2.5 g/l for V and 2 g/l for VP substrate, while glucose concentration, in the other experiments, ranged from 0.02 to 0.9 g/l in V and from 0.14 to 0.4 g/l in VP. The consortium was the second best cellulolytic performer for both substrates probably due to the contribution of the strain *Buttiauxella* sp. 4 within the consortium.

4.3.3 *Metabolic products from vegetable waste fermentation*

H_2 production is accompanied with production of Volatile Fatty Acids (VFAs) and/or solvents. The composition of VFAs generated through fermentation is an important factor, and is a useful indicator for monitoring the H_2 production pathways [50-52]. The high VFAs concentrations achieved in this study indicate that favorable conditions for the growth and the activity of acid-

producing microorganisms were established during the course of the experiments (Table 3). The detected soluble metabolites were acetate, ethanol, butyrate, propionate, formate and lactate, indicating a mixed acids fermentation type [53]. In all batch experiments the acetate was the major component (53-72%).

The differences in the metabolites concentration, produced during fermentation, showed the presence of different metabolic pathways related not only to the kind of substrate, but also to the presence/absence of inocula. The highest amount of Total Fermentation Products (TFPs) were obtained for VP substrates, both in the presence and absence of different inocula. A higher accumulation of TFPs (mg/l) at the end of fermentation resulted in a higher volumetric production of H_2 ($R^2 = 0.95$). The higher amounts (ml) of H_2 obtained with VP, compared to those obtained with V, were associated with a higher production of acetic acid and butyrate. In the fermentation of V, higher amounts of propionate were produced (Table 3).

The inocula are probably involved in the activation of two additional metabolic pathways, that further increased the overall H_2 production (Figure 2b).

Figure 2b shows the consumption of lactate as substrate of fermentation, related to the formation of elevated concentrations of butyrate at the end of fermentation. Many authors [54-56] reported that some microorganisms are able to convert lactate to butyrate, with a concomitant H_2 production. The possible reactions of lactate conversion to H_2 and butyrate [57,58] are the following:

	$\Delta G°'$, kJ/mol	
$Lactate + 0.4\ Acetate + 0.7\ H^+ \rightarrow 0.7\ Butyrate + 0.6\ H_2 + CO_2 + 0.4\ H_2O$	-183.9	(1)
$Lactate + Acetate + H^+ \rightarrow Butyrate + 0.8\ H_2 + 1.4\ CO_2 + 0.6\ H_2O$	-59.4	(2)
$2\ Lactate + H^+ \rightarrow Butyrate + 2\ H_2 + 2\ CO_2$	-64.1	(3)

All these reactions are energetically favorable, as shown by the Gibbs energy values. In our experiments it was possible to take into account the last metabolic pathway (Eq. 3) since no consumption of acetate was observed. This H_2 producing metabolic pathway was not detected in self-fermentation.

Figure 2b also shows H_2 production through degradation of formate. This reaction is catalyzed by the formate hydrogenlyase (FHL), an enzymatic complex typical of the enteric bacteria [21]). This

pathway was not observed in the inoculum with B*uttiauxella* sp.4 and in the self-fermentation: only in the former formate production was observed without consumption.

4.3.4 *H_2 production on different carbon sources in batch fermentation*

Buttiauxella sp. 4, and *Raoultella* sp. 47 and *Rahnella* sp. 10 could grow on BFM without sugars addition, with a total H_2 production of 4.03 ± 0.09, 4.70 ± 0.23 mmol H_2/l, and 9.12 ± 0.09 H_2 mmol/l respectively.

To determine the effect of the different substrates on the H_2 potential, the modified Gompertz equations [33,45] were used to fit the cumulative H_2 production data obtained from each batch experiment.

The results showed that *Buttiauxella* sp. 4, *Rahnella* sp. 10 and *Raoultella* sp. 47 were able to produce H_2 using xylose and cellobiose as the sole carbon source, but there were differences in the yields, lag times, and rates (Figure 3; Table 4). *Buttiauxella* sp. 4, and *Rahnella* sp. 10 were also able to produce H_2 on arabinose The yields reported were calculated by subtracting the relative contribution of BFM to the production of H_2.

Using cellobiose, *Raoultella* sp. 47 exhibited the best performance with a total H_2 volume of 1068 ml/l, during 48h of fermentation, corresponding to 2.84 mol H_2/mol glucose equivalent. *Rahnella* sp. 10 showed a longer lag phase and gradual H_2 production, with a total H_2 volume of 948 ml/l (2.23 mol H_2/mol glucose equivalent). Buttiauxella sp. 4 produced 748 ml/l of H_2 (in 48h) with a yield of 1.85 mol H_2/mol glucose equivalent. In *Rahnella* sp. 10 the percentage of H_2 in the biogas showed the highest values, reaching 43% on cellobiose.

Using xylose there was no significant differences between the three strains with an average yield of 1.2 ±0.02 mol H_2 /mol xylose.

As regards to final metabolic products, *Raoultella* sp. 47and *Rahnella* sp. 10 exhibited similar metabolic pathways with acetic acid and ethanol as main products on both substrates (Table 5). In Buttiauxella sp. 4 H_2 production on xylose and cellobiose produced acetic and formic acids and acetic and lactic acids as major metabolic products, respectively.

Growing on arabinose, *Rahnella* sp. 10 has shown to be a good H_2 producer with a total H_2 production, during 144h fermentation, of 1150 ml/l and a yield of 1.18 mol H_2/mol arabinose. At the end of fermentation, arabinose was completely degraded with production of acetic acid and ethanol as main soluble metabolites. *Buttiauxella* sp. 4 produced 309 ml/l of H_2 during 122h fermentation (0.28 mol H_2/mol arabinose), accompanied by 37.35% arabinose consumption and with acetic and formic acids, as main soluble metabolites. In *Rahnella* sp.10 the time course profile of the cumulative H_2 production on arabinose (Figure 3) showed longer lag phase (66h). An

intermediate lag phase occurred between 24-48h with a H_2 production of 9.1 mmol H_2/l and without arabinose consumption. This was due to the positive effect of peptone and yeast extract on H_2 production.

The H_2 yields obtained in this study were comparable to literature data (Table 6). As shown in table 6, most of the results reported in literature were obtained by adding peptone and yeast extract. Unlike other authors, the yields reported in our study were calculated by subtracting the respective contribution of BFM (containing peptone, yeast extract, L-cystein HCL, vitamins and salts) to the production of hydrogen.

Different values of H_2 conversion efficiency (%) on pentose were obtained, according to the theoretical stoichiometry considered. Several authors reported for both, facultative and obligate anaerobic bacteria, a theoretical maximum yield on pentose of 3.33 mol H_2 /mol pentose. In this case, H_2 conversion efficiency on xylose and arabinose ranged between 35-37% and between 9-35%, respectively. Kongjan et al. [70] showed that the 1.67 mol H_2/mol xylose is the highest theoretical yield when acetate and ethanol are the main metabolites. Ghosh and Hallenbeck [31] reported that in *Escherichia coli*, an anaerobe facultative bacterium with enteric type metabolisms (such as our strains), pentoses are generally degraded through the non-oxidative pentose phosphate pathway (3 pentose → 5 pyruvate → $5H_2$ + 5 CO_2 + 5 (acetate ethanol)), with a maximum theoretical yield of 1.67 mol H_2/mol pentose. In this case H_2 conversion efficiency on xylose and arabinose ranged between 71-73% and 17-71%, respectively. For hexoses, more studies agree in differentiating the maximum theoretical yields, depending on the fermentation type. Mixed acid fermentation is performed by facultative anaerobes with a maximum yield of 2 mol H_2/ mol hexose (1 hexose→ 2 pyruvate → $2H_2$ + 2 CO_2 + 2 (acetate ethanol)). Anaerobic fermentation is performed by obligate anaerobes with a maximum yield of 4 mol H_2/ mol hexose (1 hexose→ 2 pyruvate → $4H_2$ + 2 CO_2 + 2 acetate). In this study H_2 conversion efficiency on cellobiose ranged between 48-71%.

4. 4 Conclusion

The bioaugmentation of the microbial waste communities appeared to be effective in improving the biohydrogen production and turned out to be a valid alternative to expensive and energy consuming pretreatment of biomass and/or inocula. All the individual bacterial inocula promoted both, a significant increase (up to 4 times) of the H_2 yield and of the H_2 production rate (up to 2 times). A defined artificial microbial consortium was found to produce the higher specific H_2 amount (85.65 and 66.69 mL H_2/gVS respectively for V and VP) with a significant increase (3-4 times) of the rates

when compared with substrate self fermentation. Indeed the addition of indigenous bacteria populations promoted additional metabolic degradation pathways.

The three single strains were also characterized for their ability to produce H_2 on different sugars, xylose, arabinose and cellobiose, as these are key products of hydrolysis of cellulose and hemicelluloses.

Buttiauxella sp. 4, and *Raoultella* sp. 47 *Rahnella* sp. 10, are promising facultative anaerobe strains for H_2 production from the cellulosic hydrolysates and showed different behavior on different carbon sources. In particular *Rahnella* sp. 10 was the most versatile strain, showing a relatively good H_2 production on xylose (1.22 mol H_2/ mol xylose) and cellobiose (2.24 mol H_2/ mol glucose equivalent), while being the only one able to produce H_2 on arabinose. *Buttiauxella* sp. 4 showed the best cellulolytic performance and the highest H_2 yield on vegetable waste, while it was the less efficient in the H_2 production on arabinose and cellobiose.

More studies on fermentative H_2 production by pure culture, using organic waste as substrate, are recommended.

Since in our research the main aim was the increase of hydrogen yields by bioaugmentation we assessed the function (i.e. hydrogen production) as the parameter for efficiency, as it was taken into account by several authors [18,20,22,71,72]. The effectiveness of the specific axenic cultures of "indigenous" bacteria is demonstrated by the fact that the hydrogen production increases with the addition of each single strain. Co cultures of different strains increased further the performances of each single strain: this latter results show the sintrofic action between the strains. However, the analysis of microbial community structure could be useful to monitor the strain population dynamics and their ecological relationships with the microbial indigenous community. Therefore, in order to optimize the composition of the artificial consortium and also to investigate deeply the interactions hypothesized between the strains, the microbial community analysis will be performed in our future works.

4. 5 References

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4. 6 Tables and Figures

Table 1. H_2 yields, Gompertz equation parameters and Pearson correlation coefficients (R^2) between observed and predicted data for different inocula and for self-fermentation on V and VP.

Seeds	H_2 yield (ml H_2/ g VS)	Ps (ml H_2)	R' (ml H_2/h)	λ (h)	R^2
Vegetable waste (V)					
Buttiauxella sp. 4	71.27 ± 6.24	33.60	1.50	4.00	0.99
Rahnella sp. 10	47.54 ± 2.14	22.42	1.61	2.33	1.00
Raoultella sp. 47	69.7 ± 1.81	32.86	1.51	1.17	1.00
Consortium	85.65 ± 0.37	40.38	2.47	2.73	1.00
V	21.95 ± 5.35	10.09	0.58	5.89	1.00
Vegetable waste plus Potato peels (VP)					
Buttiauxella sp. 4	62.45 ± 7.75	46.39	1.55	4.00	0.99
Rahnella sp. 10	37.2 ± 0.47	27.08	1.57	1.05	1.00
Raoultella sp. 47	47.17 ± 5.08	34.34	1.27	0.00	0.99
Consortium	66.69 ± 3.25	48.55	2.14	1.11	0.99
VP	18.46 ± 0.66	13.16	0.78	5.85	0.99

Table 2 - Comparison of the Literature Data on biohydrogen production yields of anaerobic batch reactors treating waste.

Feedstock	Seed	Pretreatment inoculm	Pretreatment feedstock	Temperature (°C)	Yield (ml H_2/gVS)	Reference
Garbage slurry	Garbage slurry enrichment	NO	NO	37	31.1	[24]
Food waste	Food waste	NO	NO	35	4	[26]
Dairy manure	Dairy manure	Infrared oven 2h	0.2% HCl 30 min boiled	36	31.5	[39]
Sewage sludge	Sewage sludge	Sterilized (121°C)	Sterilized (121°C)	37	16.26	[40]
Food waste	Food waste	90 °C 20 min	90 °C 20 min	35	96.9-153.5	[26]
Vegetal waste	Vegetal waste	NO	NO	28	21.95	This study
Vegetal waste + potato peels	Vegetal waste + potato peels	NO	NO	28	18.46	This study
Potato	Anaerobic sludge	boiled 15 min	NO	37	106	[41]
Lettuce	Anaerobic sludge	boiled 15 min	NO	37	50	[41]
Wheat straw	Cow dung compost	NO	HCl 2% + microwave heating	36	68	[42]
Wheat straw	Cow dung compost	NO	-	36	1	[42]
Corn stover	Heated sludge	105°C 2h	220°C 3 min	35	49	[43]
Cabbage	Anaerobic digested sludge	100°C 15 min	NO	37	62	[44]
Carrot	Anaerobic digested sludge	100°C 15 min	NO	37	71	[43]
Rice bran	Soy bean meal	100°C 15 min	NO	35	61	[45]
Food waste	Grass compost	180*C 3h	NO	35	77	[46]
Food waste	Seed sludge	90°C 10 min	NO	35	59.2	[47]
Vegetal waste	Defined Consortium	NO	NO	28	85.65	This study
Vegetal	Defined	NO	NO	28	66.69	This study

Table 3 Metabolic products (VFAs, lactate and ethanol) of batch fermentation on V and VP (mg/l and %) at the end of fermentation.

Seeds	TFPs	Lactate		Formate		Acetate		Propionate		Butyrate		Ethanol	
	mg/l	mg/l	%	mg/l	%	mg/l	%	mg/l	%	mg/l	%	mg/l	%
Vegetable waste (V)													
Buttiauxella sp.4	3391	436	13	77	2	1969	58	277	8	317	9	315	9
Rahnella sp.10	2741	1	0	0	0	1825	54	200	6	128	4	589	17
Raoultella sp.47	3359	133	4	14	0	1776	52	415	12	277	8	744	22
Consortium	3785	487	14	53	2	2063	61	315	9	141	4	726	21
V	1910	16	0	0	0	1156	34	348	10	74	2	315	9
Vegetable waste plus Potatoe peels (VP)													
Buttiauxella sp.4	3768	128	4	176	5	2105	62	242	7	742	22	375	11
Rahnella sp.10	3350	22	1	53	2	2411	71	89	3	224	7	550	16
Raoultella sp.47	3590	24	1	89	3	2226	66	294	9	512	15	444	13
Consortium	4399	100	3	146	4	2486	73	241	7	625	18	802	24
VP	2765	40	1	0	0	1785	53	129	4	229	7	582	17

Table 4. H_2 yields, Gompertz equation parameters and Pearson correlation coefficients (R^2) between observed and predicted data for different inocula with different carbon sources.

Seeds	H_2 yield (mol H_2/ mol $sugar_{added}$)	Ps (ml H_2)	R' (ml H_2/h)	λ (h)	R^2	Substrate degradation efficiency (%)
Cellobiose						
Buttiauxella sp. 4	1.85 ± 0.13	37.20	2.20	5.47	1.00	89
Rahnella sp. 10	2.12 ± 0.17	47.40	1.66	20.68	0.99	97
Raoultella sp. 47	2.84 ± 0.07	53.44	4.25	6.73	1.00	100
Xylose						
Buttiauxella sp. 4	1.18 ± 0.07	54.62	2.39	10.14	1.00	95
Rahnella sp. 10	1.22 ± 0.07	66.17	2.43	19.86	1.00	99
Raoultella sp. 47	1.20 ± 0.11	54.96	3.35	8.31	1.00	100
Arabinose						
Buttiauxella sp. 4	0.28 ± 0.03	15.87	0.21	1.00	0.99	37
Rahnella sp. 10	1.18 ± 0.07	57.52	1.01	67.09	1.00	100

Table 5. Metabolic products (VFAs, lactate and ethanol) and H_2 percentage of batch fermentation on different carbon sources (mg/l) at the end of fermentation.

Seeds	H_2 %	Lactate (mg/l)	Formate (mg/l)	Acetate (mg/l)	Propionate (mg/l)	Butyrate (mg/l)	Ethanol (mg/l)
Cellobiose							
Buttiauxella sp. 4	28	1925	406	899	230	8	543
Rahnella sp. 10	43	48	371	1410	10	0	931
Raoultella sp. 47	38	604	76	881	0	0	896
Xylose							
Buttiauxella sp. 4	36	843	2420	1306	242	8	788
Rahnella sp. 10	40	81	294	1341	25	0	1222
Raoultella sp. 47	42	29	72	1167	11	0	791
Arabinose							
Buttiauxella sp. 4	15	82	510	528	236	7	126
Rahnella sp. 10	38	291	528	1310	518	0	884

Table 6. Comparison of batch H_2 production yields on xylose, arabinose and cellobiose from present study and literature data.

Substrate	Seed	Temp (°C)	Yield (mol H_2/mol sugar)	References
Cellobiose (glucose equivalent)	*C. termocellum*	50	1.73	[59]
	Mix	35	2.19	[60]
	Cl. acetobutylicum	30-37	2.30	[61]
	Cl. phytofermentans	30-37	1.60	[61]
	Ruminococcus albus	37	5.59	[62]
	Buttiauxella sp. 4	28	1.85	This study
	Rhanella sp. 10	28	2.23	This study
	Raoultella sp. 47	28	2.84	This study
Xylose	*Paenibacillus polymixa*	39	0.04	[63]
	Mix	36	1.92-2.25	[64]
	Clostridium amygdalinum C9	35	2.2-2.25	[28]
	Enterobacter aerogenes HGN-2	37	1.98	[50]
	Clostridium tyrobutyricum ATTC25755	37	0.77	[65]
	Clostridium butyricum CGS5	37	0.68	[66]
	Ruminococcus albus	37	2.07	[62]
	Enterobacter cloacae IIT-BT 08	36	0.95	[67]
	Buttiauxella sp. 4	28	1.18	This study
	Rhanella sp. 10	28	1.22	This study
	Raoultella sp. 47	28	1.20	This study
Arabinose	Sludge	37	1.98	[68]
	Sludge	37	1.46	[69]
	Escherechia coli DJT135	35	1.02	[31]
	Clostridium amygdalinum C9	35	1.78	[28]
	Ruminococcus albus	37	1.44	[62]
	Buttiauxella sp. 4	28	0.28	This study
	Rhanella sp. 10	28	1.18	This study

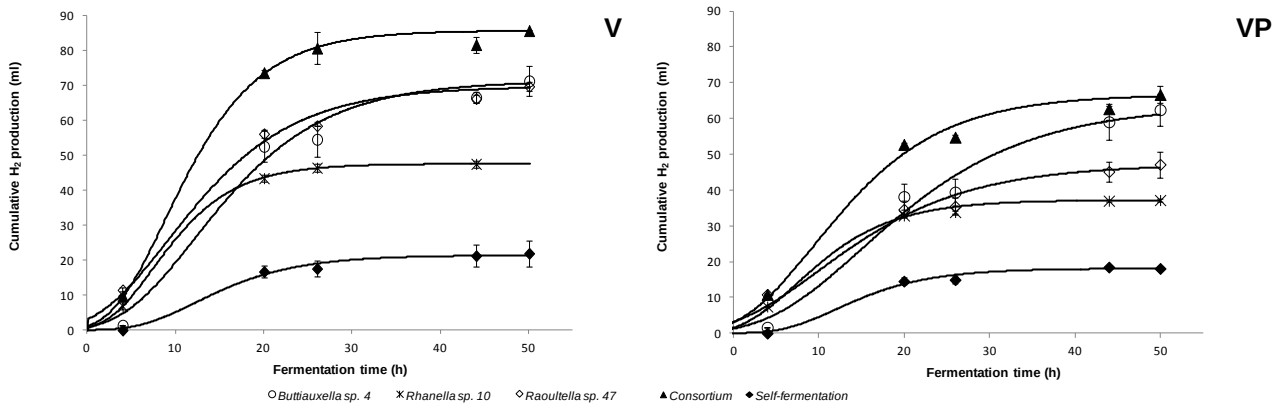

Figure 1. Cumulative hydrogen production (ml H_2/gVS) by self-fermentation and by different microbial inocula on Vegetable waste (V) and Vegetable waste plus Potato peels (VP).

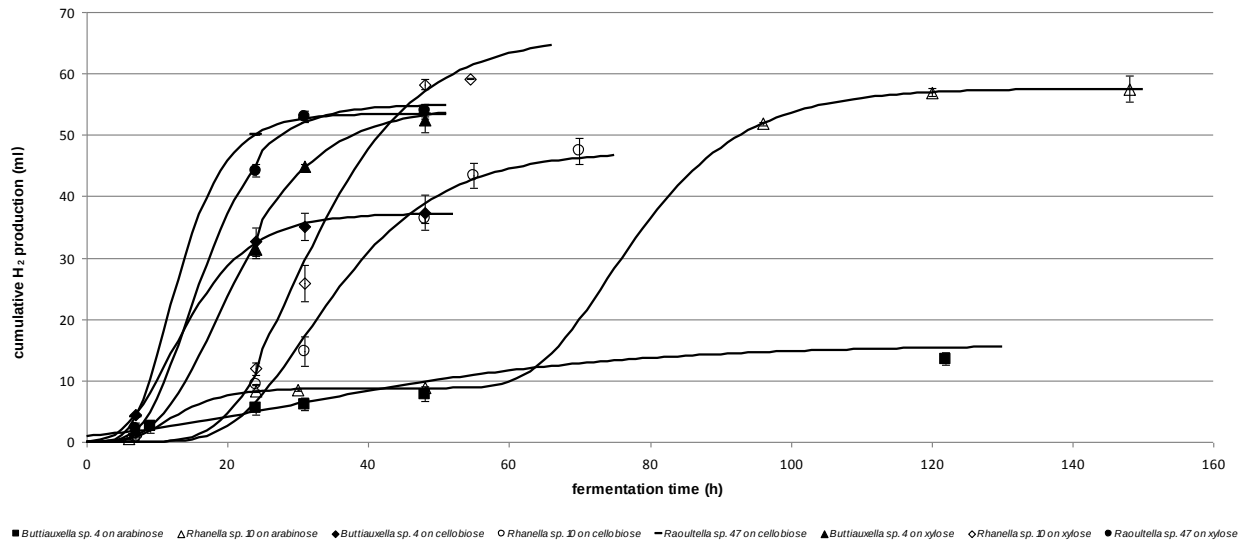

Figure 3. Cumulative hydrogen production by different inocula on different carbon sources (5 g/l).

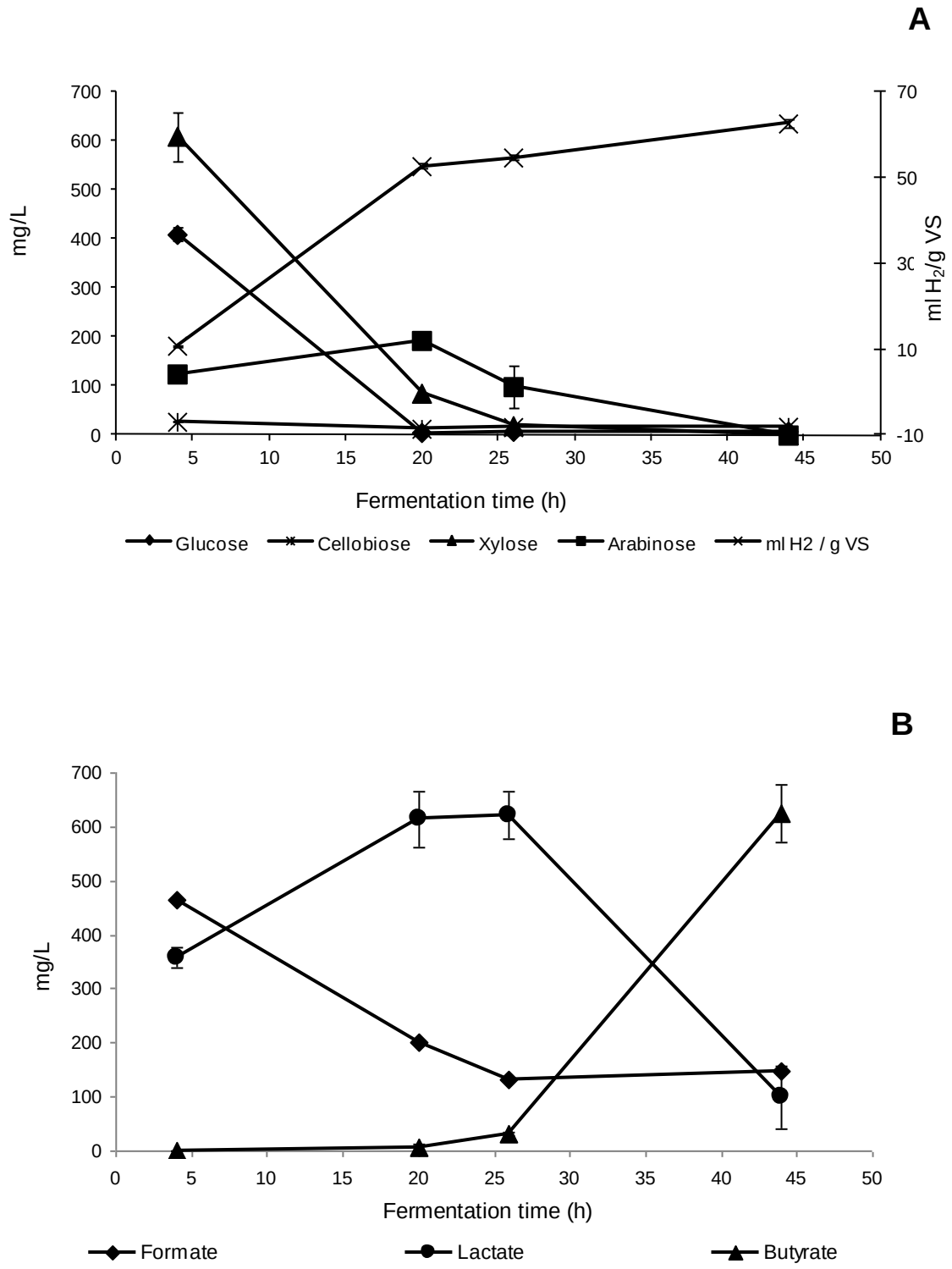

Figure 2. Time course profiling of hydrolytic products and H_2 production yield (ml H_2/g VS) (a) and of metabolic products (b) during fermentation.

5. CONCLUSIONS

This thesis showed the effectiveness of adopting an ecological approach to the study of dark fermentation towards the development of renewable and cost-effective processes for bio-H_2 production. When considered as a natural ecosystem, biowastes can serve both as feed and seed for H_2 production, therefore, the study of H_2-producing microorganisms contained in the waste could allow to tap into a great genetic diversity to select specific organisms suitable for enhancing the hydrogen production functionality of the indigenous microbial community of the waste itself.

Using this approach, by studying natural microorganisms and by manipulating the bioprocess, the required microbial metabolic capacities can be effectively enhanced from a natural environment. In addition, organic wastes are locally available and their use may contribute to reduce national dependence on imported fossil fuels.

In particular, this study demonstrated that bio-H_2 can be produced by self-fermentation of common domestic waste sources at temperatures close to ambient, only by controlling of pH.

Bioaugmenting the waste microbial community, enhances the H_2-production performance of indigenous bacteria. Thus, specific bacteria, isolated from a certain type of waste, selected and enriched for the ability to generate H_2, when re-inoculated again in their original waste, are well suited to enhance this specific functionality into the same microbial community of origin.

The practice adopted in this case, could remove the need for energetically costly pretreatments which also cause a loss of microbial diversity.

The hydrogen economy per se is still some decades away and at the present, economics strongly favour largescale hydrogen production systems. Although positive features of anaerobic hydrogen fermentation technology have been demonstrated in laboratory studies, this technology is yet to compete with those commercial hydrogen production processes from fossil fuels in terms of cost, efficiency and reliability. The results reported in this thesis, show this technology to be outstanding, geographically adaptable and potentially cost-effective, as well as suitable for decentralized power generation and for creating multifunctional bio-refineries in the short term.

The major contributions of this thesis work are summarized as follow:

1. Vegetable waste successfully served not only as a substrate, but also as a source of suitable inocula. Self-fermentation of common domestic vegetable refuses produced H_2 at temperatures of 28°C and 37°C without the need of chemical or physical pre-treatment of the substrate. Methane production was never detected. The highest yields (23.9 l/kg of VS) and H_2% were obtained at 28°C, only by controlling pH.

2. Screening microbial diversity of the waste can be a successful strategy to select highly efficient H_2-producing bacteria. 11 bacterial isolates were obtained directly from waste samples, one was identified to be member of the family *Streptococcaceae* and ten of the family *Enterobacteriaceae*. The 16S rRNA gene sequences of selected isolates have been determined and deposited onto NCBI GenBank under accession number: *Pectobacterium* sp.2 (FJ587222.1); *Pectobacterium* sp.3 (FJ587223.1); *Butiauxiella* sp.4 (FJ587224.1); *Pectobacterium* sp 5 (FJ587225.1); *Enterobacter* sp. 9 (FJ587226.1); *Rahnella* sp. 10 (FJ587227.1); *Enterobacter* sp.15 (FJ587228.1); *Raoultella* sp. 47 (FJ587229.1); *Pantoea* sp. 54 (HM627386.1); *Lactococcus* sp.56 (FJ587230.1); *Citrobacter* sp.57 (FJ587231.1). Of these, genera *Pectobacterium, Buttiauxella, Rahnella and Raoultella* were characterized as potential H_2 producers for the first time in this study.
3. The hydrogen production yields and the metabolic pathways of the isolates were affected by substrate concentration and hydrogen partial pressure. The results indicated that higher hydrogen production yields (1.58-2.20 mol H_2/mol glucose) occurred at the initial glucose concentration of 1 g/l at $pH_2 \leq 0.98 \times 10^4$ Pa. Furthermore, high pH_2 lead to a shift of metabolic pathways towards production and accumulation of more reduced substrates, such as lactate and ethanol. The high conversion efficiency of the new hydrogen producers, obtained at glucose concentration of 1g/l, make them new candidates for biological hydrogen production at low temperature (28°C).
4. Bioaugmentation of the microbial waste communities appeared to be effective in improving the biohydrogen production and turned out to be a valid alternative to expensive and energy consuming pretreatment of biomass and/or inocula. The individual bacterial inoculum of *Buttiauxella* sp. 4, *Rahnella* sp. 10 and *Raoultella* sp. 47, promoted a significant increase (up to 4 times) of the H_2 yield and of the H_2 production rate (up to 2 times). A defined artificial microbial consortium, composed of the three strains put together, was found to produce the highest specific H_2 amounts (85.65 and 66.69 mL H_2/gVS respectively for leaf-shaped Vegetable refuse (V) and leaf-shaped Vegetable refuse plus Potato peels (VP)) with a significant increase (3-4 times) of the rates when compared with substrate self fermentation. Indeed the addition of indigenous bacteria populations promoted additional metabolic degradation pathways.
5. *Buttiauxella* sp. 4, and *Raoultella* sp. 47 *Rahnella* sp. 10, are promising facultative anaerobe strains for H_2 production from the cellulosic hydrolysates and showed different behaviour on different carbon sources. In particular, *Rahnella* sp. 10 was the most versatile strain, showing a relatively good H_2 production on xylose (1.22 mol H_2/ mol xylose) and cellobiose (2.24 mol H_2/

mol glucose equivalent), while being the only one able to produce H_2 on arabinose. *Buttiauxella* sp. 4 showed the best cellulolytic performance and the highest H_2 yield on vegetable waste, while it was the least efficient in the H_2 production on arabinose and cellobiose.

In conclusion, the results of this PhD study, strengthen and deepen research lines on a methodology of H_2 production still scarcely investigated. It is pointed out that the self-fermentation process at temperatures close to ambient seems advantageous from an industrial and commercial perspective. It could be possible to improve both H_2 production and yield from fermentation of vegetal waste using autochthonous pure bacterial strains as starters.

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