Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

INGEGNERIA CHIMICA, DELL'AMBIENTE E DELLA SICUREZZA

Ciclo XXV

Settore Concorsuale di afferenza: 09/D3

Settore Scientifico disciplinare: ING-IND/25

BIOHYDROGEN PRODUCTION FROM FOOD INDUSTRY WASTE BY SUSPENDED AND IMMOBILIZED THERMOPHYLIC BACTERIA

Presentata da: JOCÉLIA DE SOUSA MENDES

Coordinatore del Dottorato

Prof.ssa Serena Bandini

Relatore

Prof. Davide Pinelli

Correlatore

Ing.Dario Frascari

Esame finale anno 2013

In nature nothing is created,

nothing is lost,

everything is transformed.

(Antoine Lavoisier)

INDEX

1. INTRODUCTION

Due to increasing population and industrial, there is consequently an increase in energy consumption, mainly derived from petroleum. This increasing consumption of energy from non-renewable sources has driven many universities and private companies to develop research seeking new energy sources that are low cost, renewable and meet the growing demand of population. The production of biofuels, including ethanol, biodiesel and biogas has gained prominence in the increasingly global market because they are derived from renewable sources by use or industrial waste, they are biodegradable and clean burning [1].

The transportation sector is the largest consumer of energy currently almost exclusively using liquid fossil fuels. The total or partial replacement of fossil fuels by biofuels is the key to minimize this crisis. One of the most promising is the production of biofuels using biomass.

Several types of biofuels are being studied and some already are being used on a small scale, such as methane, ethanol and biodiesel. Even if hydrogen is an energetic vector indeed rather than a renewable energy source, it offers enormous potential as clean energy biofuel. Hydrogen has the highest gravimetric energy density of any other fuel known. Hydrogen is regarded as the energy of the future, because of its high energy content and the fact that during its combustion not CO_2 is emitted [2].

Biological production of H_2 can be based on fermentative or photo biological processes [3].

The latter is an interesting process because of high yields of H_2 that can be obtained and the variety of substrates that can be used [4, 5].

1.1 ENERGY

A major challenge for the production of biofuels is the competition with food, many biofuels are produced from food. Data of database of global primary agricultural show that very little or nothing of the food produced is intended for the production of biofuels. However, the use of waste for the production of these biofuels could replace about a quarter of global consumption of fossil fuels for the transportation sector.

The limitations of land, water and energy are the main difficulties for the expansion production of biofuels through primary agriculture [6].

Currently the world is experiencing an energetic crisis, due to the fact that the possible use of alternative energy sources is neglected. Actually a large proportion of the

alternative sources are generated through the waste by microbial productions, so that in addition to providing clean energy they help the environment [7].

For over 30 years, oil has dominated the world economy and its derivatives have become important in several industrial sectors. The oil beyond is used to produce fuels such as gasoline, diesel oil and kerosene, and it is also the basis of many products which go from paraffin, to tissues and plastics. [8, 9]

Severe climatic changes are being accelerated by the consumption of oil and its derivatives, especially by the release of large amounts of gases which cause the greenhouse effect deteriorating the quality of our environment. In 2006 the world used 85 million barrels of oil per day and it is assumed that in 2015 it will pass to 105 barrels per day[7].

A further factor to consider is that, while the human population during the twentieth century is increased by a factor of 6, the power consumption is increased by a factor of 80 [10];the world average of energy consumption today is 2kW per capita. It is necessary, in the economy of energy, a major shift from fossil fuels to renewable energy flows [11]

The hope for a future less dependent on fossil fuels and with lower climatic problems is what has driven several universities and companies to study new ways to product renewable energies, those energies that are capable of using waste or renewable material that in short term are renewed, without undermining future generations.

These renewable energies are much talked: wind energy, solar, tidal, geothermal, biomass and energy from renewable resources in the short term. For the latter case we have: bioethanol, biodiesel and biohydrogen.

The hydrogen is a neutral energy source for CO_2, the utilization of H_2 as energetic source has a promising future and is an alternative to fossil fuels. The hydrogen can be produced starting from undesirable residues which is an advantage [12].

1.2 BIOMASSES

Biomass is by definition the total mass of living organisms in a given area; is an important energy reserve because it is composed primarily of carbohydrates. Main sources of biomass are: wood, agricultural residues, municipal solid waste, animal waste, waste from food production, aquatic plants and algae [13]. Biomass can be converted into heat for thermal use in industry, and to generate electricity, or it can be transformed into other forms of energy: solid (charcoal, briquettes), liquid (ethanol, biodiesel), and gaseous (biogas).Biomasses employed to retrieve energy are essentially energetic crops and organic

residues from forests, agriculture, farming, wastes and sewage (Figure 1.1).The use of biomass as an energy source has several advantages such as: significant reduction of emissions of greenhouse gases, reduction of the dependence from petroleum, production of liquid renewable biofuel for the transportation sector.

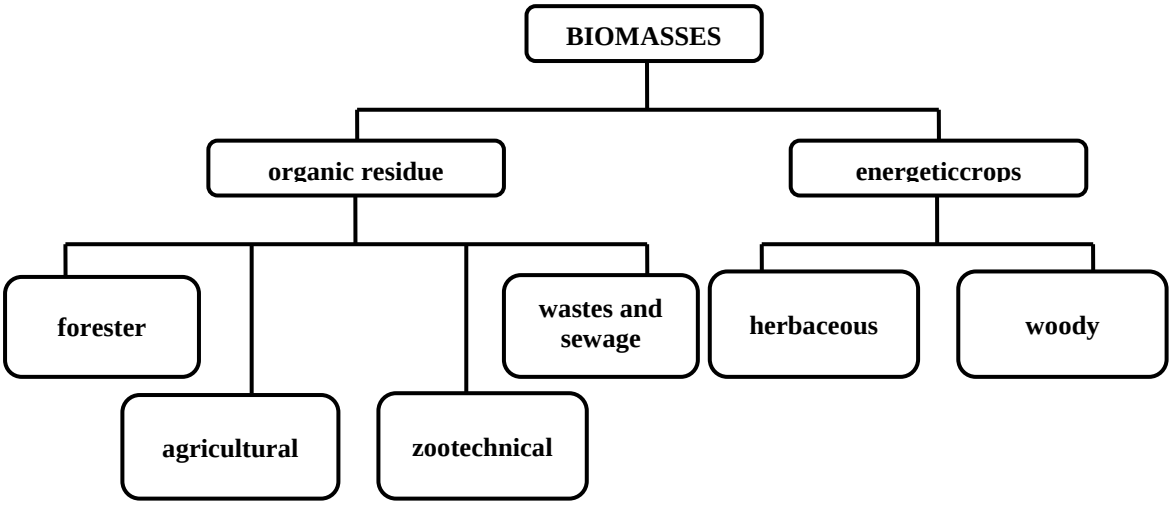

Figure 1.1 – Main sources of biomasses employed in power generating industries [13]

Biomass can be used to produce:

- ✓ **Electrical energy through the processes:**
  - ✓ ***Gasification***

The process of biomass gasification is not new, it has at least 100 years.Currently the gasification process has major interst because it produce clean energy. This process consists in the conversion of biomass into steam, which passes through a turbine connected to a generator; in this way mechanical energy is turned into electrical energy. Gasification of biomass, provides a positive impact on the environment, since all carbon dioxide produced during the process is absorbed by plants to be used again in the process. So the concentration of carbon dioxide is kept unchanged, thereby bringing a neutral carbon balance during production of electricity, contributing to reduction of greenhouse gases.

- ***Pyrolysis***

The pyrolysis process is the simplest and oldest; it involves exposition of the biomass to high temperatures (300 to 500 °C) in absence of oxygen, accelerating the decomposition of the biomass and production of a mixture of gas, liquid (vegetable oil) and solids (charcoal)

✓ **Biofuels**

- ***Ethanol***

Is a renewable fuel produced from the fermentation of biomass. Ethanol could be 1ª to 2ª generation, depending on the type of biomass used during fermentation. The ethanol is the most used biofuel, it may be used alone or in combination with gasoline in cars. The bioethanol burning is less aggressive on the environment than fuels derived from petroleum.

- ***Methanol***

Is a fuel that can be obtained by gasification of biomass. In this process, "biomass is first converted into a synthetic gas, and only then is shifted toward methanol." Most of the methanol produced is used as an industrial solvent, anti-freezer or to synthesize other substances. In the U.S., about 38% is blended with gasoline for purposes of Transportation.

- ***Biodiesel***

Is produced through the transesterification reaction of vegetable oils and animals which produce glycerin as a byproduct. It is used in cars with diesel engines.

- ***Biogas***

Are produced from the anaerobic fermentation of: vegetable waste, rotting carcasses and agroindustrial waste. This process of decomposition of organic matter by bacteria generates biogas which is a mixture of several gases, mainly methane, but it also produces CO_2 and H_2. In the Figure 1.2 scheme of production of biogas.

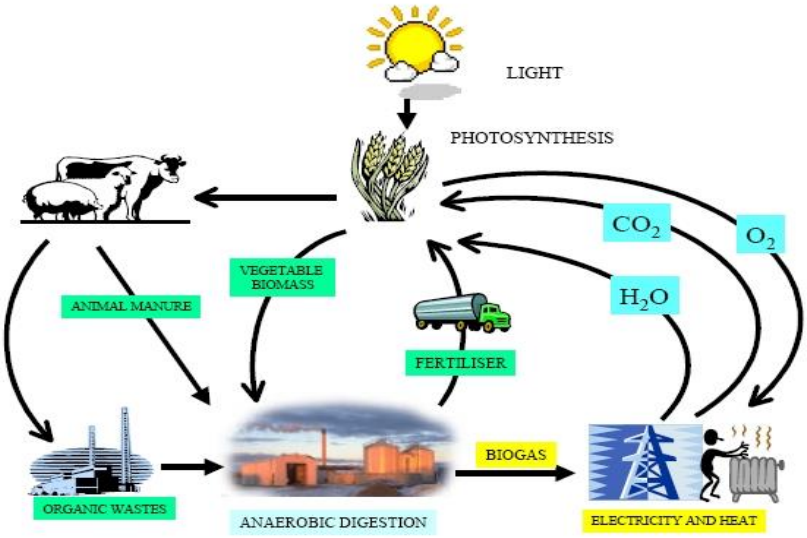

Figure 1.2 - Scheme of production of biogas [14].

The Figure 1.3 shows a summary of the processes applied to starting production of energy biomass on an industrial scale. The red dashed line represents the process used in this study.

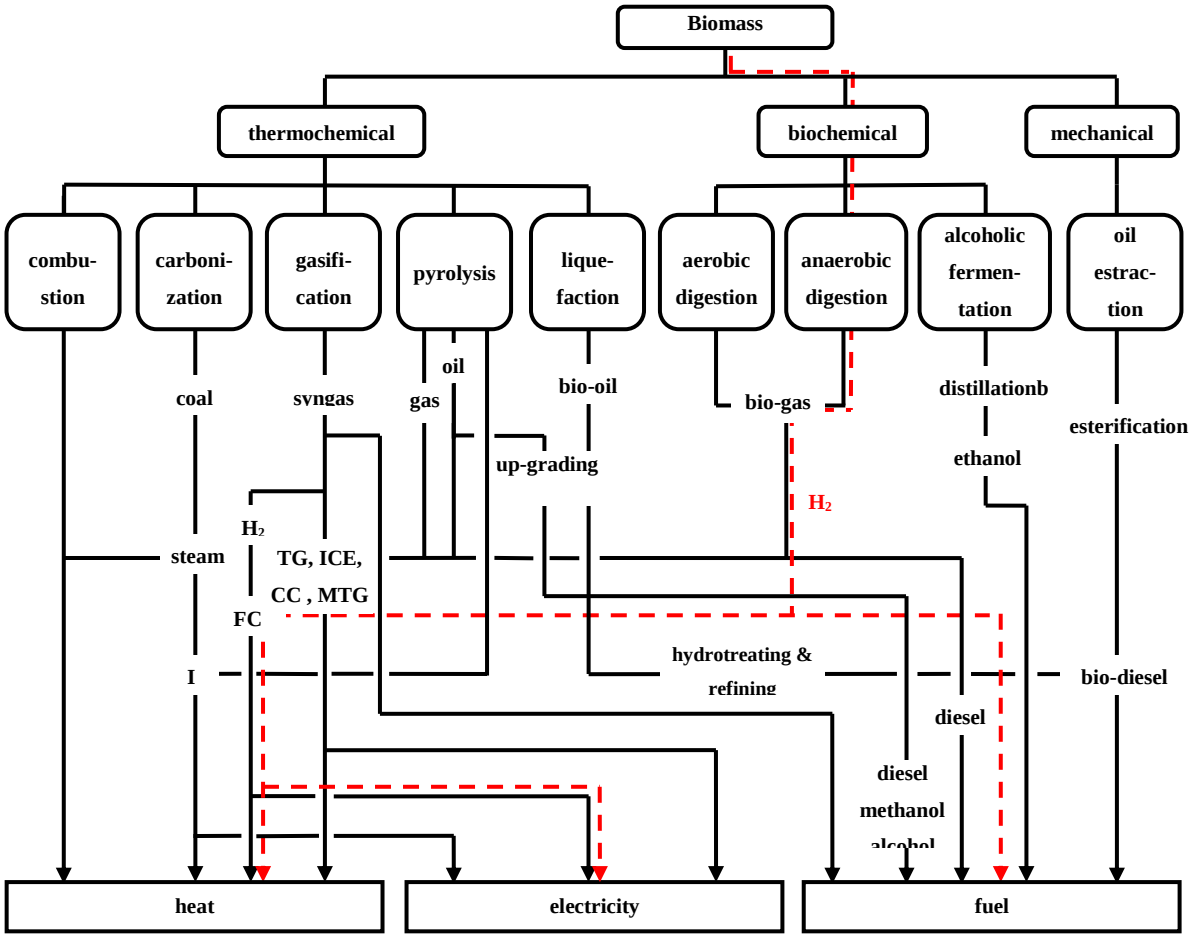

Figure 1.3 - Scheme of production of biogas [15]

1.3 HYDROGEN

Hydrogen is the most abundant chemical element in the universe constituting about 75% of the elemental mass, is represented by the symbol H, has atomic mass approximately 1.0 u, is the chemical element less dense and can be ontained in its molecular formula H_2.

Molecular hydrogen is a flammable gas, odorless, colorless, non-metal and insoluble in water. The hydrogen molecules are very light and easily escape the Earth's atmosphere into space due to this weightlessness is that the hydrogen is found in the land combined with other elements, such as: the water of the oceans, natural gas, oil, coal and biomass. The hydrogen is not a source of primary energy, but an energetic vector, an energy carrier. As an energetic vector that has great advantages due to its great efficiency in transforming its chemical energy into other forms of energy, for example in electricity.

The energy contained in one kg of hydrogen is three times greater than the energy contained in one kg of gasoline. In the table 1.1 some of industrial applications of are listed.

Table 1.1 – Application of H_2 in the industry.

Segment	Application
Energy generation	Used as a source of thermal energy in burners or in the fuel cells
Electronics industry	Used in the manufacturing process of semiconductors
Pharmaceutics industry	Used in hydrogenation of oils and fats
Metallurgical processes	Reducing agent of metallic ores
Chemical processes	Used in the manufacturing of methanol, ammonia, chlorine and caustic soda
Petroleum refining	Used for removing sulfur from fuels and in the processes hydrocracking

1.3.1 HYDROGEN PRODUCTION

The methods for hydrogen production are based on the separation of H_2 from raw materials. The raw materials dictate the methods of separation that must be applied. In Europe about 95% of H_2, equivalent to 8 million tons is produced through the reaction between coal and natural gas with steam at high temperatures; the two are fossil fuels, not renewable sources in the short term and burning release pollutants.

To obtain a high purity H_2 is used the process called electrolysis, where H_2 is produced starting from water. The electrolysis is a viable alternative and the clean technology currently dominant. H_2 can also be produced by renewable energy such as solar, hydro, wind, wave and biomass. In Table 1.2 there are the shapes of H_2 production from various sources.

Table 1.2 – Process Hydrogen production [16].

METHOD	PROCESS	RAW	ENERGY	EMISSIONS
THERMAL	Reformation	Natural Gas	High temperature steam	Carbon Dioxide
	Hydrolysis Thermochemical	Water	Heat from nuclear energy	Without emissions
	Gasification	Coal, biomass	Steam, oxygen, heat and pressure	Some emissions
	Pyrolysis	Biomass	Vapor average temperature	Some emissions
ELECTRIC	Electrolysis	Water	Electricity	Depending on the primary energy used
	Photoelectrochemical	Water	Sunlight	Without emissions
BIOLOGICAL	Fotobiológico	Water and algae	Sunlight	Without emissions
	Anaerobic digestion	Biomass	Heat	Without emissions
	Fermentation	Biomass	Heat	Some emissions

✓ **THERMAL**

- ***Reforming***

The reform of the natural gas is one of the most efficient Hydrogen productions generating a recovery of 70 to 80% conversion. The first step of the reaction decomposes the fuel into carbon monoxide and H_2. A subsequent reaction converts carbon monoxide and water to carbon dioxide and hydrogen. The disadvantage of this process is the release of carbon dioxide into the atmosphere, contributing to the pollution of the planet. In industry scale is currently the dominant method to produce H_2.

- ***Thermochemical hydrolysis***

Water spontaneously dissociates at around 2500°C, but this thermolysis occurs at temperatures too high for usual process piping and equipment. Catalysts are required to reduce the dissociation temperature.

- ***Gasification***

Gasification is the method that converts coal in synthetic fuel of direct application in energy production. The disadvantages of this process are the major environmental impacts and risks for the workers in the mills, processing problems such as fire hazards and human exposure to carcinogens and to high temperatures.

- ***Pyrolysis***

Pyrolysis is a thermochemical decomposition of organic material at elevated temperatures without the participation of oxygen. It involves the simultaneous change of chemical composition and physical phase, and is irreversible. The word is coined from the Greek-derived elements pyro "fire" and lysis "separating".

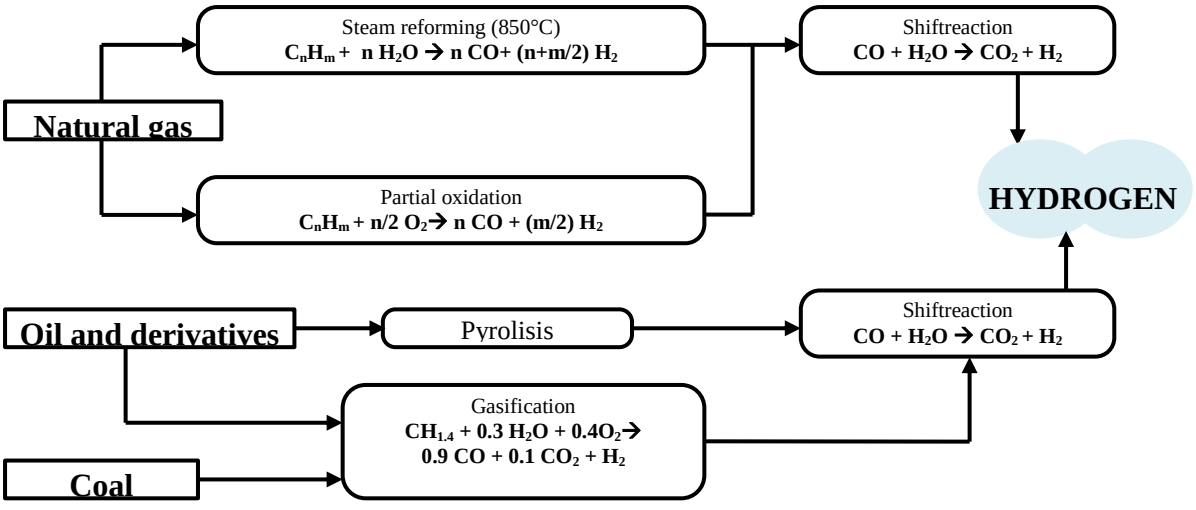

Figure 1.4 – Summarized of Thermal process Hydrogen production [15].

✓ **ELECTRIC**

- ***Electrolysis***

Is a chemical process not spontaneous caused by an electric current, which promotes a chemical reaction, converting electrical energy into chemical energy. The H_2 can be produced through the electrolysis of water, which consists of passing an electric current in the water, thus producing hydrogen and oxygen; this process is clean and relatively inexpensive, but still not as diffuse as the H_2 production of fossil fuels. In Figure 1.5 is an example of Hydrogen production by electrolysis.

Currently less than 5% of the world's hydrogen is generated from the electrolysis of water. This is due to the cost of electricity, equivalent to two-thirds of operating costs, which makes the process very expensive when compared to other methods.

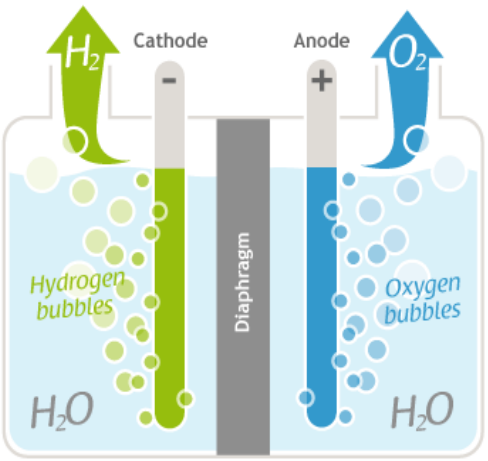

Figure 1.5 – The water electrolysis [17].

- ***Photoelectrochemical***

The photoelectrochemical production of Hydrogen occurs in 2 ways. One of the methods uses hydrosoluble metal complexes as catalysts, these metallic complexes dissolves absorbing solar energy and producing an electrical charge that initiates the decomposition reaction of water. The other method uses semiconductors in a photochemical cell and converts electromagnetic energy into chemical. The semiconductor surface has the function of absorbing solar energy thereby acting as electrodes. The disadvantage of this process is the amount of energy expended to produce hydrogen, often greater than the energy provided by the hydrogen produced.

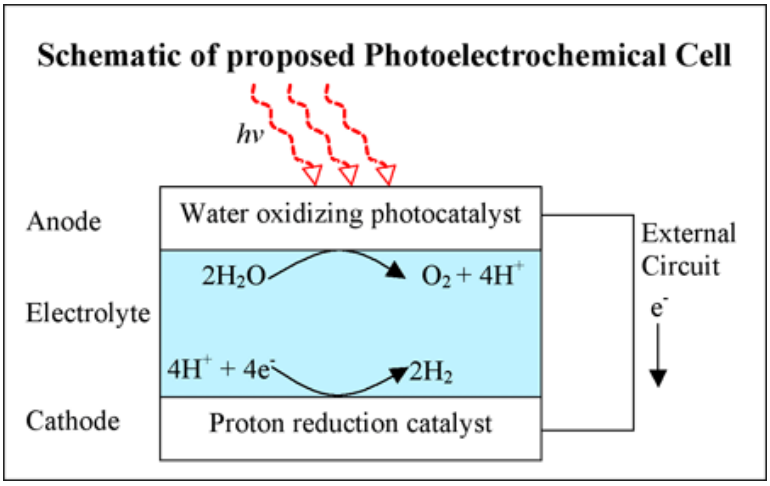

Figure 1.6 – photoelectrochemical cell [18].

✓ **BIOLOGICAL**

- ***Photosynthesis***

The photosynthesis is the conversion of water, sulfur compounds or organics compounds in the hydrogen, through the sun radiation. Photosynthesis is the process that uses sunlight to convert water, sulfur compounds and organic compounds in hydrogen. This production can occur in two ways, through the biophotolysis and photodecomposition.

1. Biophotolysis

Biophotolysis sets up the action of sunlight on a biological system that results in the decomposition of a substrate, usually water, producing hydrogen. The biophotolysis can be direct or indirect. The general equation which represents the biophotolysis is:

$$2\ H_2O\ +\ \text{light} \longrightarrow 2\ H_2\ +\ O_2$$

Direct Biophotolysis

This process uses green algae to convert solar energy into chemical energy in the form of H_2. The green algae have an efficient mechanism that produces H_2 starting from water. For the production of H_2, first is needed a period of adaptation of algae under anaerobic conditions in the dark, in this period the enzyme hydrogenase is activated. The hydrogenase is responsible for the production of H_2, the hydrogenase combines protons (H^+) and electrons (e^-) thus forming and releasing H_2.

However, under normal conditions wherein oxygen is the byproduct of photosynthesis, hydrogen production cannot be maintained for more than a few

minutes, because oxygen is a potent inhibitor of Fe-hydrogenase enzyme which catalyzes the process of releasing H_2. The inactivation of the enzyme occurs by the reaction of oxygen with iron located in the catalytic center of the enzyme. This process is attractive because it uses solar energy and a readily available substrate, the water, and converts it into oxygen and hydrogen. The disadvantage is the sensitivity of the enzyme Fe-hydrogenase in presence of oxygen.

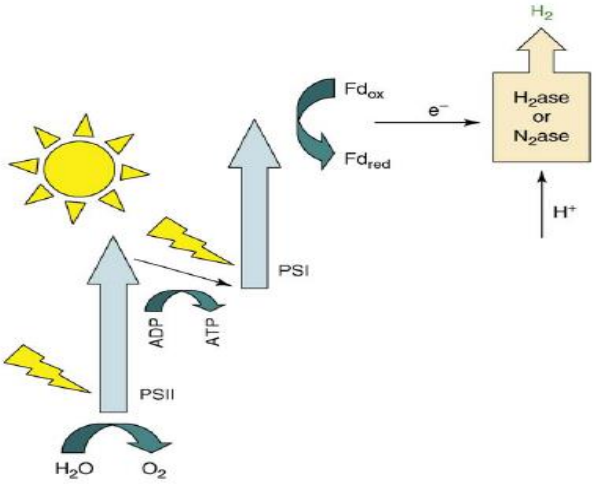

Figure 1.7 – Direct Biophotolysis [2].

Indirect Biophotolysis

The indirect biophotolysis occurs in two steps. In 1st step the CO_2 fixed in carbohydrate of accumulated for microorganisms, in the 2nd step this carbohydrate is converted to H_2; 2nd step occurs in anaerobic conditions and in photobioreactors or dark fermentation in tanks. In the equations below reactions to biophotolysis indirect.

$$12\ H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2\ (1°\ step)$$

$$C_6H_{12}O_6 + 12\ H_2O \rightarrow 12\ H_2 + 6CO_2\ (2°\ step)$$

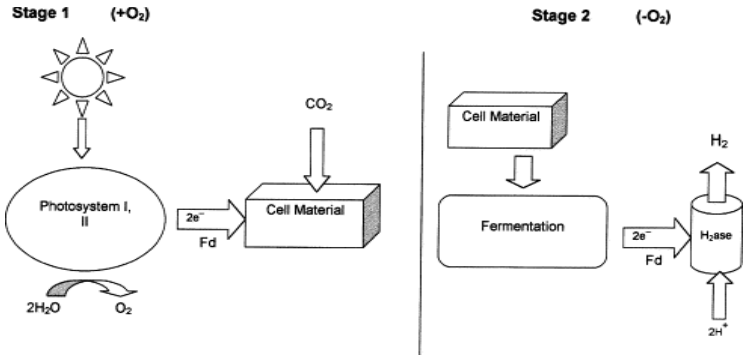

Figure 1.8 - Indirect Biophotolysis [19].

The cyanobacteria are the most studied organisms in indirect biophotolysis. These microorganisms can have 3 types of enzymes in their metabolism, they are: nitrogenase, assimilation hydrogenase and bidirectional hydrogenase.

Nitrogenase is responsible for catalyzing the reduction of nitrogen releasing Hydrogen mandatory.

Assimilation hydrogenase recycles this Hydrogen released by nitrogenase.

The bidirectional hydrogenase can function both to produce Hydrogen as to consume it.

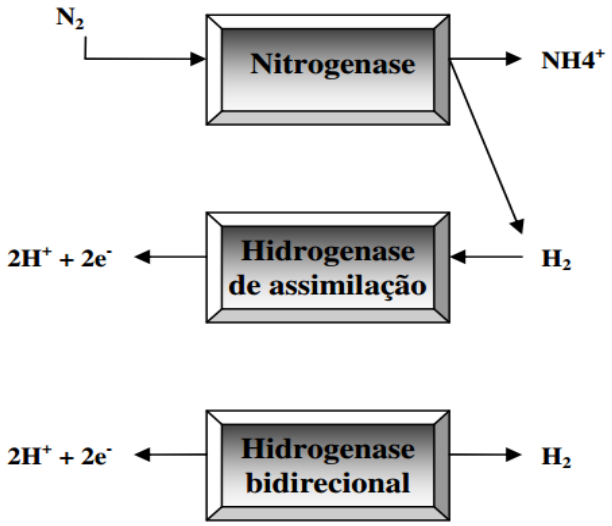

Figure 1.9 - Enzymes of indirect biophotolysis [20, 21].

Thus, for the efficient production of hydrogen, the research is aimed at producing and selecting mutants deficient in assimilation activity of H_2 and mutants whose bidirectional hydrogenase is less sensitive to oxygen. There are also several studies for optimization and process efficiency related to nitrogenase. This enzyme requires a high amount of ATP, which decreases its effectiveness in the conversion of solar energy. Thus, has pursued a nitrogenase with lower energy requirements.

2. Photodecomposition

The Photodecomposition (Figure 1.10) is a process in which organic compounds such as acetic acid, are converted into H_2 and CO_2 through the sunlight; it can be performed by non-sulfur purple bacteria that puts into place a photosynthesis anossigenica. A disadvantage of this process is the need of the enzyme to have extra

metabolic energy in the form of ATP that generates lower yields of H_2 per unit of absorbed light.

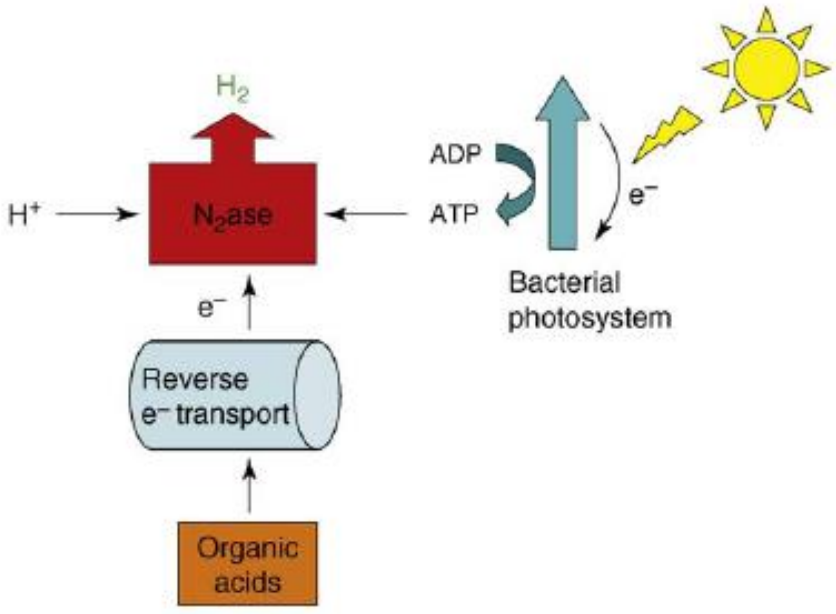

Figure 1.10– Photodecomposition [2].

Examples of photodecomposition bacteria are: Rhodopseudomonas palustris, Rhodospirillum rubrum, and Rhodobacter spheroides. These organisms retrieve high energy electrons to reduce ferredoxin from organic compounds, usually organic acids. Solar energy is captured to produce ATP and obtain electrons through a reverse electron flow. Nitrogenases catalyzes proton reduction to molecular hydrogen [2]. For instance, rates of hydrogen production by photoetherotrophic bacteria are higher when the cells are immobilized on solid matrix. Rates of H_2 production by Rhodobacter spheroides GL1 immobilized on activated glass are 3.6-3.8 ml H_2/ml/h, that would correspond to 0.145-0.153 mmol H_2/l/h with a proper scale-up, considering ml to mol conversion at room temperature and atmospheric pressure [22]. This process can reduce drastically organic compounds in wastes treatment; however, as for photolysis, light conversion efficiency is low and photobioreactors are expensive; moreover, nitrogenases need high quantities of metabolic energy [2].

- ✓ ***Dark fermentation***

In fermentation processes hydrogen is produced by the action of enzymes hydrogenases as a means to eliminate excess electrons generated during degradation of an

organic substrate. In most microorganisms hydrogen is produced by the anaerobic metabolism of pyruvate, formed during the catabolism of most organic substrates.

Dark fermentation is carried out by a variety of different microbes that break-down anaerobically carbohydrate rich substrates to hydrogen, carbon dioxide and other organic compounds by Embden-Meyerhof pathway (Figure 1.11). This is a fermentation process that is based on simple reactor technologies; indeed, a wide range of waste streams or energy crops can be used and it does not need solar energy as photolysis and photofermentation.

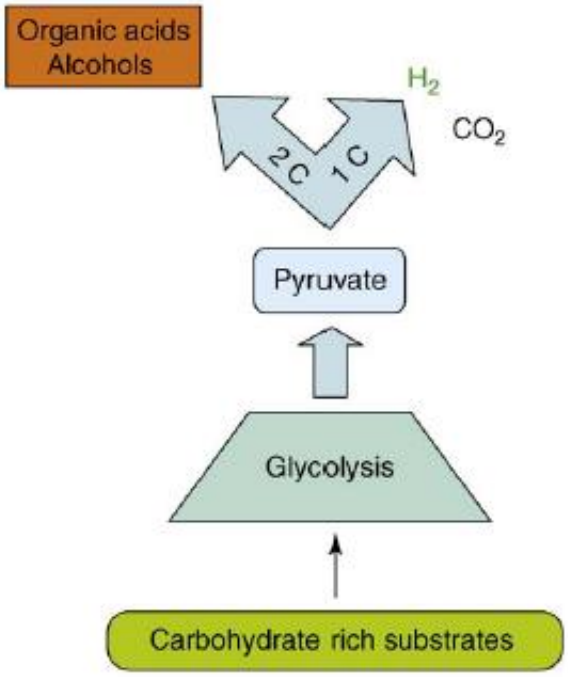

Figure 1.11 – Dark fermentation [2].

The production of H_2 by fermentation begins with the breakdown of glucose molecules, this process is equal to microorganism facultative and strictly anaerobic. Through the glycolytic pathway, glucose is converted to pyruvate; starting from pyruvate several different routes may be used thus forming the final products of fermentation.

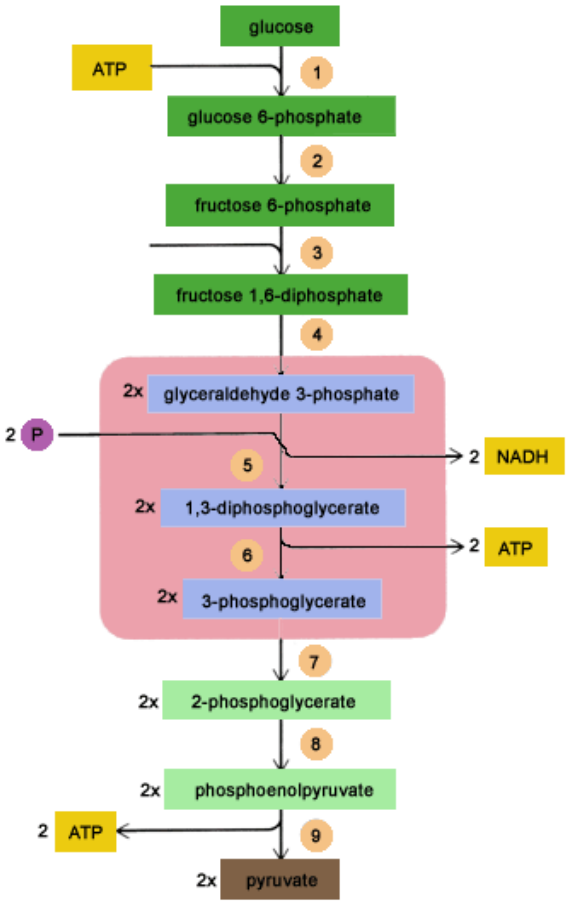

Figure 1.12 – Glycolysis [23].

Pyruvate is the key intermediate key in the principal catabolic pathways for hydrogen production through glycolysis. Two different enzymes are involved in the breakdown of pyruvate, depending on the metabolic type of fermentation: pyruvate formate lyase (Pfl) in enteric-type mixed-acid metabolism and pyruvate:ferredoxin oxidoreductase (Pfor) in clostridial-type metabolism. In both pathways acetyl-CoA can be converted into acetate through an acetyl-phosphate intermediate that drive ATP synthesis.In the mixed-acid fermentation of enteric bacteria (Figure 1.13), pyruvate is cleaved to acetyl-CoA and formate without the immediate production of reducing equivalents. In point of fact there is no energetic advantage for the bacterium to produce hydrogen unless a pH drop, caused by active metabolism, switch on the Fhl system (formate: hydrogenlyase). Thus, proton reduction coupled with formic acid oxidation mitigate acidification. Acidic conditions also promote pyruvate conversion into lactic acid, with the formation of one molecule of acid instead of two, acetate and formate. By the way, reductants are channeled away from the

hydrogen production pathway. Since two molecules of formate are produced from each molecule of glucose, the maximum theoretical yields can be predicted to be $2H_2$/glucose [24].

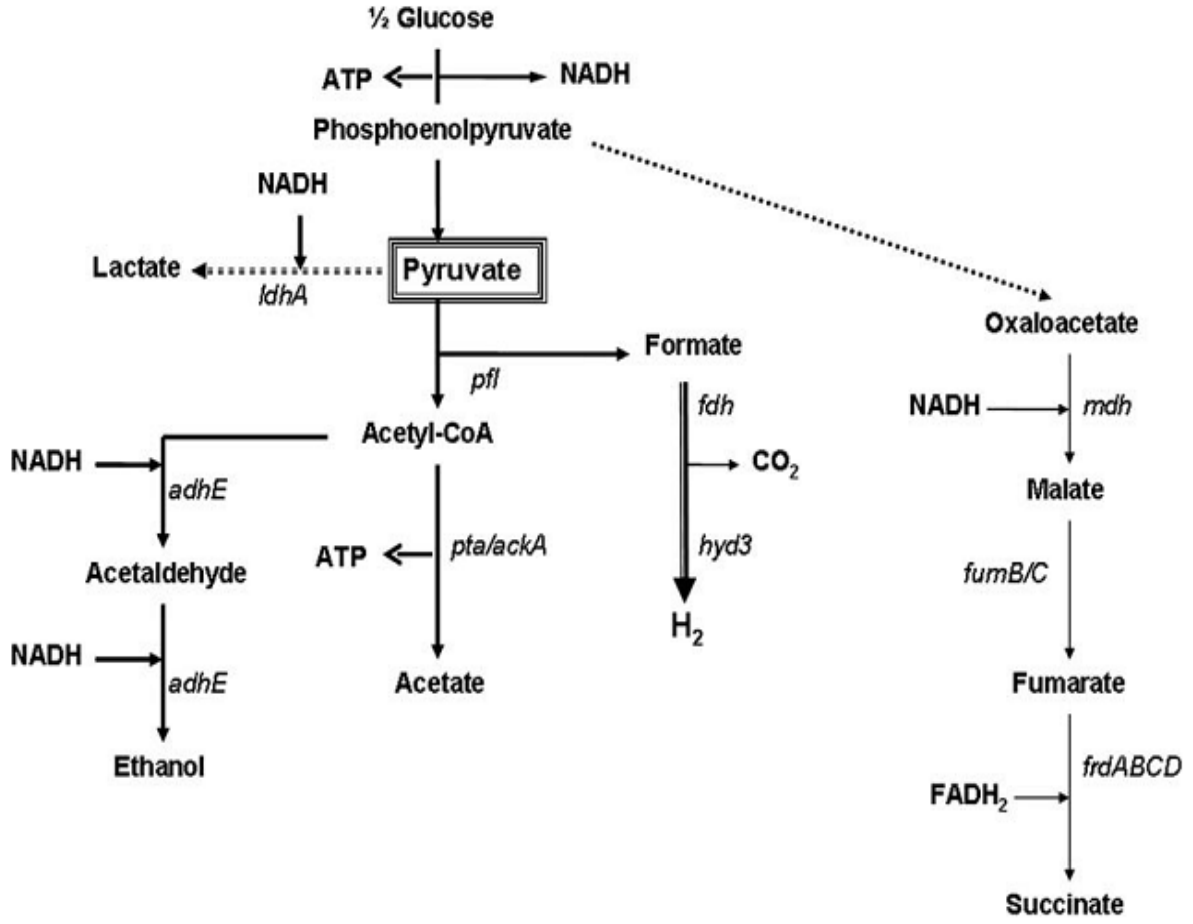

Figure 1.13 -Catabolic pathways for hydrogen production through glycolysis [24].

In facultative anaerobes, such as *E. Coli*, the pyruvate is converted to formate and acetyl-CoA, the reaction being catalyzed by an enzyme complex called pyruvate-formate lyase (PFL). The formate is then rapidly degraded by the enzyme-formate hydrogen lyase (FHL) releasing CO_2 and H_2 [24]. Because only two molecules can be produced from each two molecules of pyruvate, these microorganisms present as yield most 2 moles of H_2 per mole of glucose. Several factors may still influence this yield, reducing it, for example, the production of lactate from pyruvate, which will compete with the reaction production hydrogen.

In obligate anaerobes, such as *Clostridium sp*. the pyruvate is converted into acetyl-CoA and CO_2 by pyruvate-ferredoxin oxidoreductase (PFOR) with a transfer of protons and electrons to ferredoxin (Fd). By the action of hydrogenase, the Fd releases hydrogen [25]. Thus, two moles of hydrogen are obtained per mole of glucose. In addition, two other moles

of H_2 can be produced using the NADH generated during glycolysis and ferredoxin oxidoreductase (NFOR), totaling a maximum yield of 4 moles of H_2 per mole of glucose (Figure 1.14). However, in practice this efficiency is never reached.

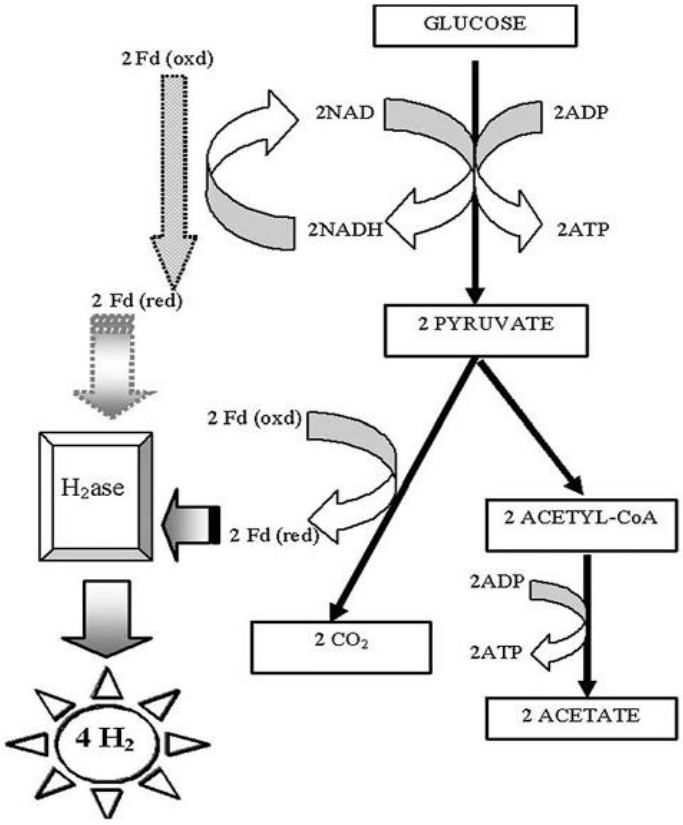

Figure 1.14 – Clostridial-type hydrogen producing fermentation [24].

Low yields and production rates represent major hurdles to practical application of biohydrogen technologies. In particular, in dark fermentative hydrogen production the major barrier to face is a direct consequence of the thermodynamics of metabolic process that drive hydrogen producing bacteria to molecular hydrogen evolution metabolism: 2 H_2 mol/glucose (enteric bacterial mixed acid fermentation) or 4 H_2 mol/glucose (clostridia-type fermentation) [5]. Hence, hydrogen yields are limited by reduced byproducts that cause the reoxidation of cofactor, i.e. NADH, to continue glycolysis or thermodynamic constraints.

1.3.2 BIOHYDROGEN PRODUCTION AT HIGH TEMPERATURES

High temperatures in fermentative processes can give general benefits, such as lower viscosity of solutions and media, better mixing, less risk of contamination, faster mass transfer kinetics and no need for reactor cooling. In addition, several studies demonstrated the

spread hydrolytic capacity of fermenting thermophilic bacteria and a comparative study of three model extremophiles (*Pyrococcus furiosus, Thermotoga maritima and Caldicellulosiruptor saccharolyticus*) showed a wide variety of transferase and glycoside hydrolases [26]. Polysaccharides degradation, as hydrogen formation from carbohydrates, is enhanced at higher temperatures by thermodynamics. Furthermore, species of hyperthermophilic Thermococcales and Thermotogales showed a lower oxygen sensitivity than other strict anaerobes [27]. In general, thermophilic enzymes are interesting stable proteins that could be introduced in synthetic in vitro pathway for hydrogen production in reactors. Finally, higher temperatures inhibit the growth of H_2-consuming methanogenic mesophilic microorganisms usually present in biomasses [28].

The most significant limitation of thermophilic and hyperthermophilic microorganisms is the maximum biomass level achievable, that is lower than mesophilic microorganisms, reflecting in lower H_2 productivities. Thus, to be competitive hydrogen producers, higher biomass must be obtained to take full advantage of H_2 yields close to the Thauer limit[24, 28, 29]. In addition, although sequenced genomes are available for several hyperthermophilic and thermophilic microorganisms, the majority of them are presently not accessible for genetic engineering and microbiology techniques (e.g. Petri dishes) are not completely exploitable at high temperatures. For this reason, methods are at relatively early stage of development; however, encouraging results regarding selection techniques and developed genetic tools have been achieved [30-33].

Thermophiles and hyperthermophiles are microorganisms belonging to Bacteria and Archaea domains that have an optimal temperature of growth above 60°C and 80°C, respectively[34]. Many representatives of these diverse groups of microorganisms are strictly anaerobes capable of fermentative hydrogen production. Examples of hydrogen producing thermophilic and hyperthermophilic microorganisms are shown in Table 1.8 [35].

Table 1.3 – Overview of thermophilic and hyperthermophilic hydrogen producing microorganisms [35].

Organism	Domain	Temp. grown (°C)	Culturing type	Substrate	Reported end products	H_2/ hexose
Thermophiles						
Thermoanaerobacterium saccharolyticum YS485	B	55	Batch	Cellobiose	Acetate, lactate, ethanol	0.87
Thermoanaerobacterium thermosaccharolyticum PSU-2	B	60	Batch	Starch	Acetate, ethanol, butyrate	2.8
Clostridium thermocellum ATCC 27405	B	60	Chemostat	α-Cellulose	Acetate, lactate, ethanol, formate	1.65
Hyperthermophiles and extreme thermophiles						
Thermotoga elfii DSM 9442	B	65	Controlled batch	Sucrose	Acetate	3.3
		65	Batch	Glucose	Acetate	3.3
Thermotoga neapolitana DSM 4359	B	80	Batch	Glucose	Acetate, lactate	2.4
		80	Controlled batch	Glucose/ xylose	Acetate, lactate	3.3
		85	Batch	Glucose	Acetate, lactate	3.8
		77	Batch	Glucose	Acetate, butyrate	3.2
Thermotoga maritima DSM 3109	B	80	Batch	Glucose	Acetate	4
Caldicellulosiruptor saccharolyticus DSM 8903	B	70	Controlled batch	Sucrose	Acetate, lactate	3.3
		72	Controlled batch	Glucose/ xylose	Acetate, lactate	3.4
Thermoanaerobacter tengcongensis JCM11007	B	75	Batch	Starch	Acetate, ethanol	2.8
		75	Batch	Glucose	Acetate	4
Thermococcus kodakaraensis TSF100	A	85	Chemostat	Starch	Acetate, alanine	3.3
Pyrococcus furiosus DSM 3638	A		Chemostat	Maltose	Acetate, butyrate	2.9
		90	Batch	Cellobiose	Acetate, alanine	2.8
		90	Batch	Maltose	Acetate, alanine	3.5
		90	Chemostat	Maltose	Acetate, alanine	2.6
		90	Chemostat	Cellobiose	Acetate, alanine, ethanol	3.8

Among the different thermophilic hydrogen producers, dissimilarities between metabolic pathways of substrate degradation and reductant disposal mechanism can be found. Furthermore, differences can also be found in hydrogenases cellular location and enzymatic characteristics, hydrogen productivities and hydrolytic abilities. Model organisms from different orders of Bacteria and Archaea shown in Table 1.6 and Table 1.8 are described below. The order thermotogales was chosen for this study for hydrogen production, they are extremophiles capable of producing hydrogen from waste. Below we have the description of this order.

- ✓ ***THERMOTOGALES***

The term Thermotogales identifies a family of extremophile bacteria that grow between 60 and 80°C. the order of Thermotogales include a group having extremely thermophilic characteristics: rod-shaped, non-sporeforming bacteria with an outer sheath as envelope also known as "toga" which is visible, preferably in the exponential growth phase,

as we can see in Figure 1.15 [36]. Among *Thermotogales*, the genus *Thermotoga* has recently received more attention because of genomic and metabolic versatility, which includes a wide range of enzymes involved in diverse carbohydrate utilization pathway (several monosaccharydes, α-linked and β-linked oligo- and polysaccharides), and their relative high H_2 productivities [37]. In addition, *Thermotogales* in general are deep-branched microorganisms that could be related to common ancestors that lived early on Earth. Indeed, many examples of lateral gene transfer (LGT) between *Thermotogales* and Archaea was demonstrated [38]. The genome of 6 members of this genus was sequenced: *Thermotoga maritima*, *T. neapolitana*, *T. petrophila*, *T. naphtophila*, *T. lettingae*, and *T. sp. RQ2* [39-41].

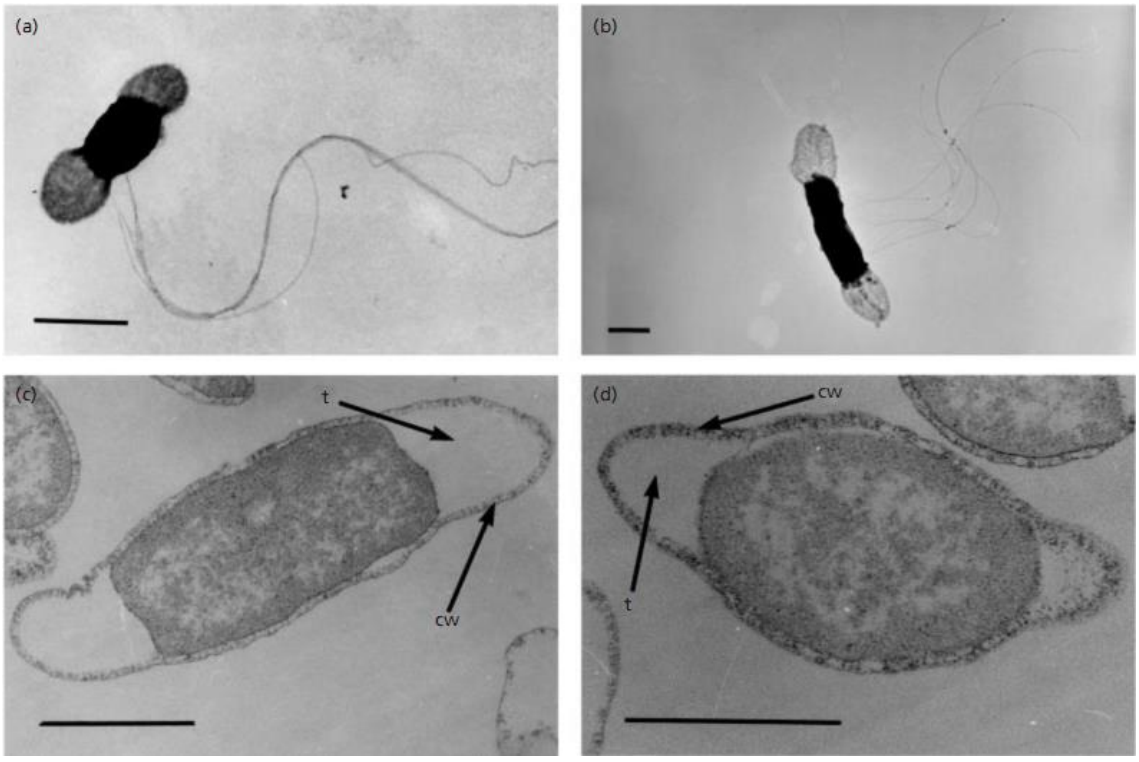

Figure 1.15 –(a)–(b) Electron micrographs of negatively stained cells of *Thermotoga petrophila* (a) and *Thermotoga naphtophila* (b). (c)–(d) Electron micrographs of doubly stained ultrathin sections of cells of *T. petrophila* (c) and *T. naphtophila* (d). Arrows indicate the toga (t) and cell wall (cw). Bars, 1 μm [36].

For our study we used bacteria of the genus Thermotoga, the bacteria carry out pathway Embden-Meyerho glycolysis, resulting: acetate, ethanol, lactate, butyrate, CO_2 and H_2. For *Thermotoga neapolitana* this pathway was demonstraded through 2D NMR-spectroscopy [42]. In Figure 1.16 we can see description of the glycolytic pathway for *T. maritima*.

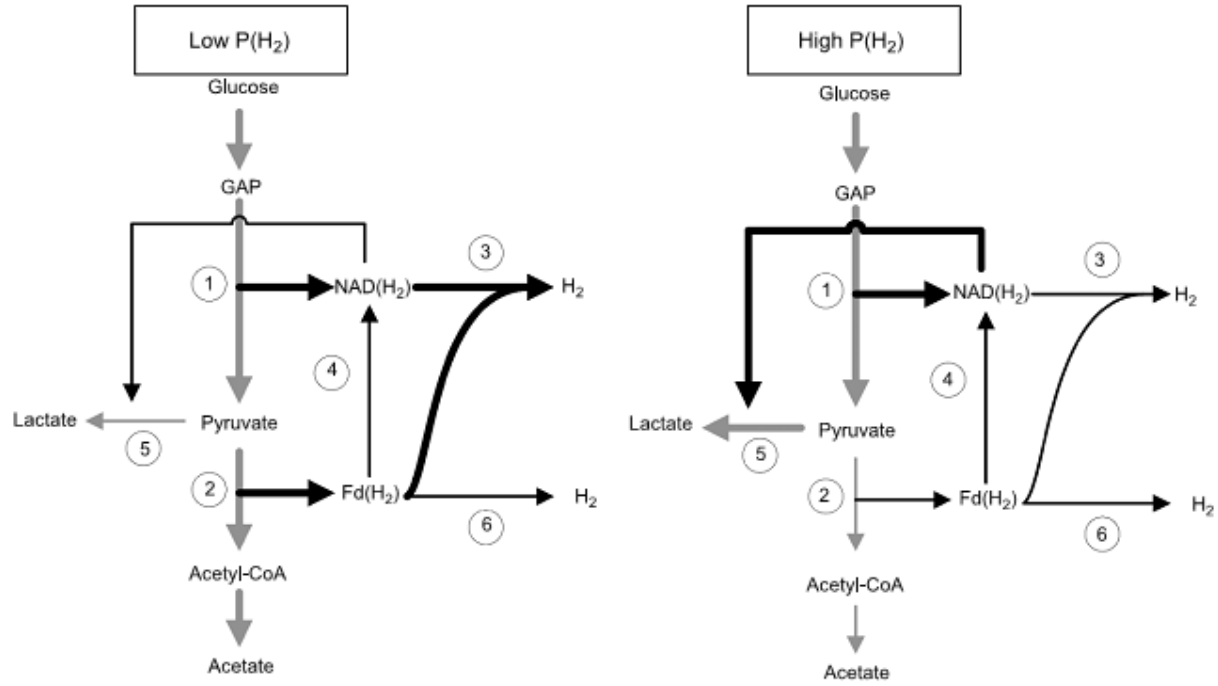

Figure 1.16 – Scheme of carbon flow (grey arrows) and electron flow (black arrows) during glucose fermentation by *T. maritima* at low and high $P(H_2)$. 1, glyceraldehyde-3-P dehydrogenase (GAPDH); 2, pyruvate:ferredoxin oxidoreductase (POR); 3, bifurcating NADH/ferredoxin-dependent hydrogenase; 4, NADH:ferredoxin oxidoreductase (Nfo); 5, lactate dehydrogenase (LDH); 6, ferredoxin-dependent hydrogenase [35].

Recently, an innovative reducing equivalent recycling mechanism by the trimeric *T. maritima* [FeFe] hydrogenase was described. It is so called *bifurcating hydrogenase* and it apparently uses the exoergonic oxidation of ferredoxin to drive the unfavorable oxidation of NADH in a 2:1 molar ratio to produce hydrogen. To maintain the 1:1 ratio of reducing equivalents, *T. maritima* seems to use a NADH:ferredoxin oxidoreductase, but so far this enzyme has not been identified. Homologues of the bifurcating [FeFe] hydrogenase of *T. maritima* have been found in several microorganisms, whose metabolism is involved in hydrogen evolution. Among *Thermotoga* species, only *T. petrophila* seems to possess a homologue of this hydrogenase [43]. The hydrogen evolution mechanisms in this order of thermophilic bacteria still needs further research efforts.

Finally, *T. neapolitana* was demonstrated to exhibit diauxic growth in medium containing 0.3 mM glucose and 1.0 mM lactose; however, cAMP levels ranged between 44 and 280 mol/mg protein, irrespective on the carbon source used. Thus, *T. neapolitana* displays a cAMP-indipendent mechanism for catabolite repression like Gram-positive bacteria[44].

All members of the order Thermotogales have demonstrated the ability to produce hydrogen; however, some members of this order stand out producing considerably greater quantities than others [27, 36, 45-46]. *T. neapolitana* in particular can completely convert glucose to CO_2, acetate and H_2in an approximate ratio 2:2:4[47]. Among Thermotogales members, *T. neapolitana* has been intensively characterized for hydrogen production: *T. neapolitana* showed so far the highest H_2 production rates [27], comparable with Thermococcales and Thermo anaerobacteriales, and H_2 yields close to the theoretical limit of 4 H_2/glucose. In Table 1.9 studies about fermentative hydrogen production by *T. neapolitana* are summarized. H_2 production rates of 14.5 mmol/L/h on glucose and 13.1 mmol/L/h on Miscanthus polysaccharide-rich hydrolisate were achieved in 1 liter batch reactor. *T. neapolitana* is a moderately halophilic organism, it tolerates conditions with higher ionic strength[48]. Thus, considering also *T. neapolitana* broad substrate range and hydrolytic abilities [27, 37, 46, 48] it can be considered a robust microorganism for biohydrogen production from processed biomasses and carbohydrate rich substrates [46, 48].

Table 1.4 – Fermentative hydrogen production conditions in batch cultures by *Thermotoga neapolitana* cited in literature. [(a)] yields are reported in mol H_2 produced/mol glucose consumed; [(b)] these results are obtained under N_2 sparging conditions; [(c)] hydrogen productivities in mmol/l/h was calculated from values reported in brackets considering that 1 mole of an ideal gas occupy 22.4 liters of volume at 25°C.

T	Batch volume	Buffer	Substrate	Organic acids	H_2/glucose yields [(a)]	H_2 productivities	Ref.
70°C	160 ml bottles	Phosphate4 mM TRIS 10 mM	Glucose (5.0 g/l)	-	-	0.651 mmol/l/h [(c)] (14.58 ml/l/h)	[49]
70°C	160 ml bottles	Phosphate 4 mM	Glucose (5.0 g/l)	-	Above 4	-	[27]
80°C	160 ml bottles	Phosphate 40 mM	Glucose (5.0 g/l)	Acetate Lactate	3.5-3.8	-	[50]
75°C	120 ml bottles	Phosphate 4 mM	Glucose (7.5 g/l)	Acetate	1.84	0.580 mmol/l/h[(c)] (440 ml H_2/l/34h)	[51]
77°C	160 ml bottles	PIPES 20 mM	Glucose (2.5 g/l)	Acetate Lactate	3.85	0.860 mmol/l/h	[47]
80°C	2 l reactor	MOPS 50 mM	Glucose (10.0 g/l)	Acetate Lactate	3.3	14.5 mmol/l/h	[48]
80°C	2.4 l reactor	TRIS 10 mM	Glucose (5.0 g/l)	Acetate Lactate	3.85	2.27 mmol/l/h [(c)] (51 ml H_2/l/h)	[42]
77°C	120 ml bottles	Phosphate 4 mM	Glucose (7.5 g/l)	Acetate Butyrate	3.24 [(b)]	0.97 mmol/l/h [(c)(b)] (1742ml/l/80h)	[52]
80°C	3 l reactor	Phosphate40 mM	Glucose (7.5 g/l)	Acetate Lactate	3.8 H_2/glucose	1.24 mmol/l/h	[46]

The defined growth media for hydrogen fermentation by *T. neapolitana* require microelements and vitamins added at adequate concentrations [53]; a reducing agent (e.g. cysteine) is also supplemented for an optimal microbial growth [50]. Defined growth media are supplied with complex nitrogen sources (e.g. yeast extract, tryptic soy broth, or peptone); yeast extract best concentration was found to be 4 g/L if added as sole complex nitrogen source for *T. neapolitana* and 6 g/L for *T. maritime* [51]. Moreover, yeast extract contributes for a final 12-15% to the H_2 produced in batch cultures of *T. neapolitana* on defined media with glucose as main carbon source[47]. Glucose best concentration for H_2 production in 120 ml serum bottle batch cultures was demonstrated to be 7.0-7.5 g/L for *T. neapolitana* [51,52] and 7.0 g/L for *T. maritime* [51].

H_2 can have an inhibitory effect on hydrogen fermenting bacteria, in particular in clostridial-type fermentation. Accumulation of H_2 has a clear inhibitory effect on cellular biosynthesis, triggers stationary phase, and, hence, limits biomass production in *T. maritime*[28]. Interestingly, *T. neapolitana* was reported to be more H_2 tolerant (25-30% in batch cultures head-space) than *T. maritima*, although reasons are still unclear [49, 50]. Not continuous N_2 sparging of head space applied when inhibitory concentration of hydrogen are achieved was reported to rise H_2 yield by 78% in *T. neapolitana* batch cultures [52]. Finally, in closed-system batch cultures, head space-culture volume ratio influences H_2 accumulation and % concentration, hence, it can contribute to a possible product inhibition. Nguyen et al. [52] demonstrated that the best fermentation system contains 40 ml of liquid volume in a 120 ml-bottle.

Van Ooteghem and colleagues supposed to achieve higher H_2 production in batch cultures by *T. neapolitana* with 6% of O_2 in head-space [27, 49]. Nevertheless, it was demonstrated that oxygen exposure in microaerophilic conditions results in lower H_2 production yields in T. neapolitana batch cultures [47, 50].

1.3.3 FACTORS INFLUENCING FERMENTATIVE HYDROGEN PRODUCTION

The fermentative production of hydrogen is a complex process influenced by many factors, such as: pH, inoculum, substrate, Hydraulic retention time (HRT), temperature and nitrogen source.

- ✓ **pH**

pH is one of the most important parameters that influence the activity of bacteria producing hydrogen, the variation of pH during fermentation can affect the action of hydrogenase and the metabolic route [54].

Dark hydrogen fermentation produces great amounts of organic acids and pH can easily drop down of 3 or 4 orders of magnitude. Several studies found out most favorable initial pH in batch cultures, even though continuous fermentation approach gives back more accurate data about the pH optimum of hydrogen production.

- ✓ **Inoculum**

To reach a high yield in the production of H_2, it is necessary to prevent the growth of microorganisms methanogens, these microorganisms consume Hydrogen, so are undesirable for the production of Hydrogen.

There are several studies that attempt to block the development of these cultures through the manipulation of some parameters, such as: operating at low pH, low temperature and short retention time cell [55, 56].

Pretreatment methods reported to suppress hydrogen-consuming activity include heat-shock, acid or base addition, aeration, freezing and thawing, chloroform addition, sodium 2-bromo-ethanesulfonate, sodium 2-bromo-ethanesulfonic acid and iodopropane. As complex communities, their composition can vary over time: a possible way to overcome this issue is designing a defined microbial consortia, a community of diverse members contributing with different metabolic capacities[57]. However, little is known so far about the complex interactions that occur between members of a consortium or how stable synthetic microbial communities can be.

- ✓ **Substrate and HTR**

In several studies it is reported a range of substrate concentration optimum that enable higher hydrogen production preventing substrate inhibition. A number of substrate was studied for fermentative hydrogen production, including sludge, agricultural and zoo technical organic waste, cellulose and lignin from residual crops, cornstalk cereals, municipal sewage[54].

Most of the studies were conducted in batch mode due to its simple operation and control. This has led to systems with more robust and long periods stable performances. However, large-scale operations would require continuous production processes for pratical engineering reasons. Most common continuous reactor types are

CSTR (continuous stirred tank reactor), UASB (up-flow anaerobic sludge blanket) reactor and retention-cell reactor. The latter is capable of maintaining higher biomass concentration and could operate at shorter hydraulic retention time (HRT). The major advance of these reactors over CSTR is their greatly increased production rate. Cell retention is obtained by granules, flocs or biofilms and many variations of this reactor type have been studied (Table 1.5), thus the comparison among processes carried out in different reactor configurations and operational parameters is difficult[2, 54].

Table 1.5 – Continuous reactor configurations for biohydrogen production [2].

Microorganisms	Substrate	Type of reactor	H_2 rate (l H_2/l/h)
Sludge (wastewater treatment plant)	Molasses	Continuous stirred-tank reactor (CSTR)	0.20
Sludge (wastewater treatment plant)	Glucose	Anaerobic sequencing batch reactor (ASBR)	0.23
Sludge (wastewater treatment plant)	Sucrose	Fixed bed bioreactor with activated carbon (FBBAC)	1.2
Activated sludge and digested sludge	Glucose	Anaerobic fluidized bed reactor (AFBR)	2.4
Sludge (wastewater treatment plant)	Sucrose	Upflow anaerobic sludge blanket reactor (UASB)	0.27
Anaerobic sludge	Sucrose	Polymethymethacrylate (PMMA) immobilized cells	1.8
Sludge (wastewater treatment plant)	Sucrose	Carrier-induced granular sludge bed (CIGSB)	9.3
Sludge (wastewater treatment plant)	Sucrose	Fluidized bed reactor (FBR)	1.4
Sludge (wastewater treatment plant)	Glucose	Anaerobic fluidized bed reactor (AFBR)	7.6 biofilm; 6.6 granules
Sludge (wastewater treatment plant)	Sucrose	Continuously stirred anaerobic bioreactor (CSABR)	15.0
Heat-treated soil	Glucose	Membrane bioreactor (MBR)	0.38

- ✓ **Nitrogen, phosphate and metal ions**

An appropriate nitrogen level is essential for growth of hydrogen-producing bacteria and thus for hydrogen productivity. The most widely investigated nitrogen source is ammonium salts; by the way also rich nitrogen sources such as peptone, tryptic soy broth (TSB) or yeast extract were analyzed. Phosphate acts as buffer in hydrogen-producing bacteria growth media and it is necessary as macroelement for nucleic acid synthesis. It has been shown that C/N and C/P ratios are fundamental for process optimization. Best values reported are 50÷200 for the former and 500÷1000 for the latter [54].

According to inoculum and substrate, hydrogen-producing bacteria need micro- and trace-element in order to maintain membrane potential and other physiological characteristics. Furthermore they play a role for protein structure and activity. Especially Fe and Ni are very important cofactors involved in hydrogenases, which are the redox enzymes able to evolve molecular hydrogen from electrons and protons [54].

✓ **Temperature**

The temperature is another factor that influences the activities of fermentative microorganisms producing hydrogen. Have been demonstrate that in the proper temperature range the organism can increase its ability to produce Hydrogen, however very high levels of temperature significantly reduce the activity of hydrogen production.

1.3.4 HYBRID TWO-STAGE SYSTEMS

The basic principle of a hybrid two-stage system is to extract additional energy in the second stage reactor from the organic acid rich effluent of a dark fermentation hydrogen producing first reactor. The second-stage coupling is advantageous to overcome thermodynamic limits of dark fermentation, by recovering energy from organic compounds of the first stage waste matter and by decreasing COD content. Three different systems are theoretically capable of complete energy extraction and they are under active investigation in laboratory-scale experiment up to pilot-scale experiment (Fig 1.17) [2].

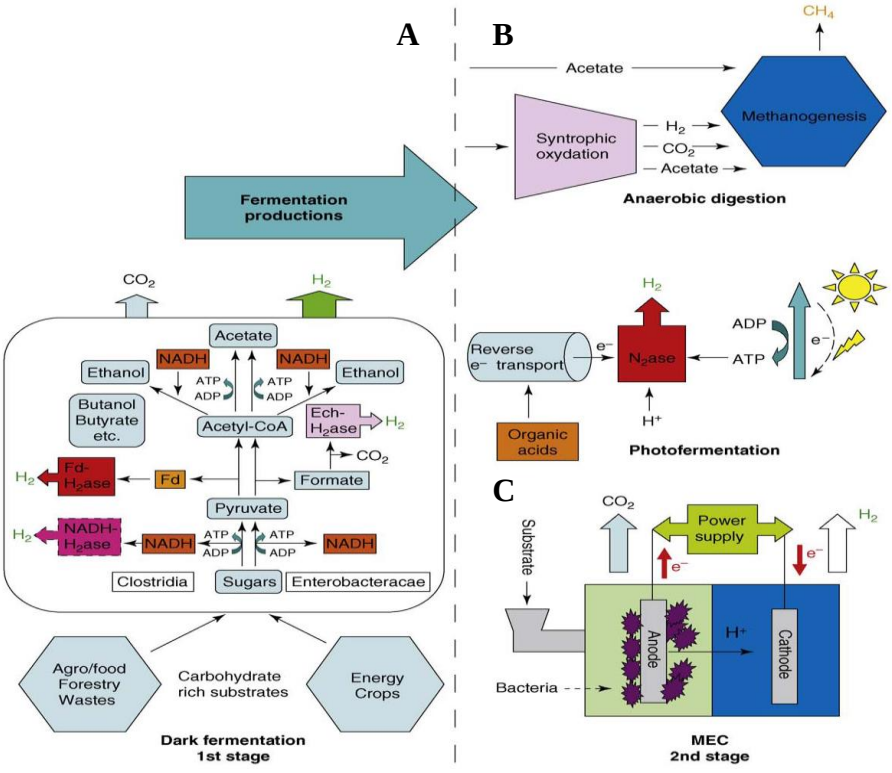

Fig. 1.17 – Different possible two-stage systems for complete conversion of substrate. The first stage (on the left) is a clostridial type or enteric bacterial type dark fermentation from carbohydrate rich substrates. Second stages (on the right) include methanogenesis (B), photofermentation (C) or microbial electrohydrogenesis cells, MCE (D) [2].

In Figure 1.17 B dark fermentation is coupled with a methanogenic second stage. The disadvantage of generating a double gas stream could be useful because hydrogen-methane mixtures are cleaner fuels for internal combustion engines rather than methane alone [2]. Although in the long term the goal should be to produce only hydrogen stream, this hybrid two-stage system has already scaled up to the pilot plant stage [58]. In this study the plant was able to provide relatively high rates of hydrogen production (5.4 L/L/d) and methane (6.1 L/L/d) while achieving an 80 % removal of the COD. The presence of the first dark fermentation reactor doubled methanogens productivities compared with a single stage methane producing process[58].

A second approach to retrieve energy from first stage effluent is *photofermentation* (Figure 1.17 C). Although in theory this metabolic process is able to complete conversion of organic matter into hydrogen, in practice as stated previously photofermentation suffers of low light conversion and low rate of hydrogen production and requires high cost transparent and hydrogen impermeable photoreactors [2].

Another possible process employs microbial *electrohydrogenesis* cells (MECs). MEC is a system in which electricity provides energy to bacterial cells able to transform organic acids into hydrogen (Figure 1.17 D). Microbial communities in the anodic chamber is versatile, thus is in theory capable to completely decompose organic effluent from the first stage reactor. Therefore, cells use the anode as terminal electron acceptor. Supplementary voltage (>200 mV) is added to that already generated from the substrate to drive hydrogen evolution at the cathode. This hydrid system could in theory achieve 12 H_2/glucose with a small investment of electric power. However, hydrogen production rates are very low and they have been obtained so far only at high applied voltages (~ 800 mV) [2].

1.3.5 HYDROGEN STORAGE

Storage is considered by many the Achilles' heel in the way of the Hydrogen Society.

The problems for the storage of hydrogen are because of some of its physical-chemical characteristics, which are: very low energy requiring large volume reservoirs and low bulk density energy storage hinders the appropriate quantities for most applications in spaces reasonably small.

Currently there are three basic choices as regards the storage of hydrogen:

• Hydrogen can be compressed and stored in high-pressure tank

• Hydrogen can be cooled until it reaches the liquid state and then stored in tanks adequately insulated

• Hydrogen can be stored in materials or substances.

Hydrogen storage systems are shown in Figure 1.18.

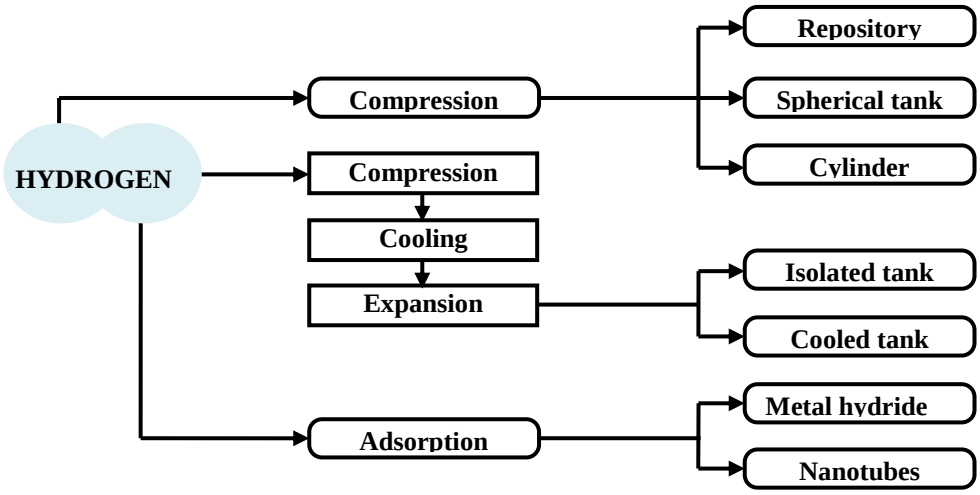

Figure 1.18 –Hydrogen storage methods [15]

Hydrogen stored as gas needs to be pressurized to 150-700 bar. Low power density per volume unit is reflected in tanks with high mass/volume ratio and in high energetic costs of pressurization, that reach 8% of the power tank content, instead of 2% for methane. Liquid hydrogen is obtained by cooling hydrogen to 20 K under pressure. This procedure is energy consuming and economically expensive since liquid hydrogen tank must be maintained at very low temperature. Adsorption storage methods are based on metal hydride or carbon nanotubes and at the current state of art they are under active development[3].

1.3.6 APPLICATION OF HYDROGEN

The hydrogen as an energy source has several applications. It can be used as: fuel for engines, industrial processes and syntheses, internal combustion engines and fuel cells. Below we have a detailed explanation about the fuel cell.

- ✓ ***HYDROGEN FUEL CELLS***

Hydrogen fuel cells and related hydrogen technologies provide the essential link between renewable energy sources and sustainable energy services [5]. Fuel cell (FC) is a promising technology of hydrogen employment. Fuel cells carry out the reverse electrochemical reaction of electrolysis with H_2 as reducing agent, O_2 as oxidizing agent and a electrolyte containing membrane as trigger. H_2 molecule is split

in electrons and protons generating an electric current (Figure 1.19). A FC provides an efficiency of power conversion from 40% up to 70%. Modularity is a very important feature of FC: connection of several fuel cells increases power supply limit of the systems. FC can also tolerate partial loads, reaching their maximum activity at 25% of load with a small decrease increasing the load up to 100%. Depending on H_2 production processes and FC technology, the only byproduct of FCs is heat. Thanks to the heat that may be produced by FCs, important applications are cogeneration and hydrogen-based engine[15].

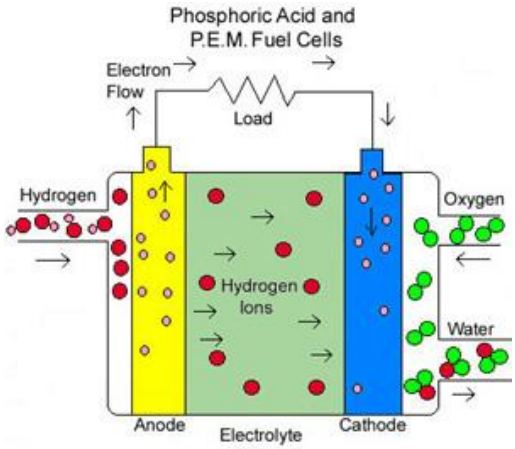

Figure 1. 19 – FC operating diagram

Problems related to FCs are high economic costs, sustainable H_2 production, low power density resulting in high plants volumes, small-medium dimensions power plants [15]. However, basing on the idea that biohydrogen producing systems might be used as small, distributed power systems at communities level, if these systems would able to deliver electricity through a FC 24 h per day, for the whole year and heat houses by co-generation they could have a useful and commercial application [5].

Levin and colleagues [5] carried out an analysis of fermentative hydrogen production to roughly estimate bioreactor volumes to power PEM fuel cell of 1kW, 1.5 kW, 2.5 kW, and 5 kW, shown in Table 1.6. An average electrical load for houses in the temperate zone is in the order of 1.5 kW for non-electrically heated houses and 2.5 kW for electrically heated houses. Electrical demands vary on diurnal and seasonal bases, and there are large differences between regions, so a 5 kW PEMFC was considered to provide sufficient power to meet the peak of demand of an electrically heated house, even producing excess energy for long periods.

Table 1.6 – Required size bioreactor to power PEM fuel cells of diverse output with different biohydrogen producing systems [5]

$BioH_2$ System	H_2 synthesis rate (reported units)	H_2 synthesis rate (converted units)
Direct photolysis	4.67 mmol H_2/l/80 h	0.07 mmol H_2/(l × h)
Photo-fermentation	4.0 ml H_2/ml/h	0.16 mmol H_2/(l × h)
Dark-fermentations		
Mesophilic, pure strain[a]	21.0 mmol H_2/l l/h	21.0 mmol H_2/(l × h)
Mesophilic, undefined[b]	1,600.0 l H_2/m^3/h	64.5 mmol H_2/(l × h)
Mesophilic, undefined	3.0 l H_2/l/h	121.0 mmol H_2/(l × h)
Thermophilic, undefined	198.0 mmol H_2/l/24 h	8.2 mmol H_2/(l × h)
Extreme thermophilic, pure strain[c]	8.4 mmol H_2/l/h	8.4 mmol H_2/(l × h)

Dark fermentation seems to have good potential to develop as practical biohydrogen producing systems. For the higher production rate of 121 mmol H_2/L/h a bioreactor of approximately 500 L (495 in the Table 1.6) would provide sufficient power for a 2.5 kW PEMFC: this order of magnitude is a reasonable volume for commercial application[5].

1.4 BIOFILMS

Biofilms are complex communities of microorganisms attached to surfaces or associated with interfaces [59]. Biofilms are generally described as consisting of the cells of microorganisms immobilized at a substratum, attached to a surface, and frequently embedded in an extracellular polymer matrix of microbial origin [60].

The formation of a biofilm begins with the anchoring of microorganisms freely floating at a surface, which initially adhere to the surface through weak and reversible forces of Van der Waals forces. the anchoring of the first settlers facilitate the arrival of other cells making various sites of cell adhesion and begin the construction of the matrix that allows the integrity of the biofilm. Once colonization has begun, the biofilm grows through cell division and integration of external bacteria, including other species. Biofilms represent a huge potential in industrial applications, medical and green biotechnology, which tends to use biological techniques to reduce, eliminate environmental pollution.

Since the 70s it was realized that a physiological important condition in the life cycle of bacteria, is represented by the formation of cell aggregates that are defined biofilm. The first studies date back to the '30s and '40s. In nature, bacteria exist in the form of aggregates, consortia multi-cell in which the cells adhere to each other [61].

The formation of the biofilm depends on aspects of chemical, physical and biological. Biofilms formation begins with the transport of molecules forming a film, a process that occurs rapidly when in aqueous medium. The formation of biofilms depends on the type of surface to which the biofilm is adhered. After the formation of the biofilm, the growth is strong influenced by fluid dynamics of the aqueous medium by gravitational forces and Brownian motion. The next step is the adherence of other bacteria through the adsorption usually pretty irreversible due to exopolysaccharide (EPS). Mature biofilms then develop by the attraction of more planktonic bacteria and entrapment of inorganic and organic molecules and microbial products, developing a complex consortia within which there is physiological cooperation between different species. This results in increased heterogeneity and the development of chemical micro gradients within the biofilm [60]. In the Figure 1.20 there is the biofilm life cycle.

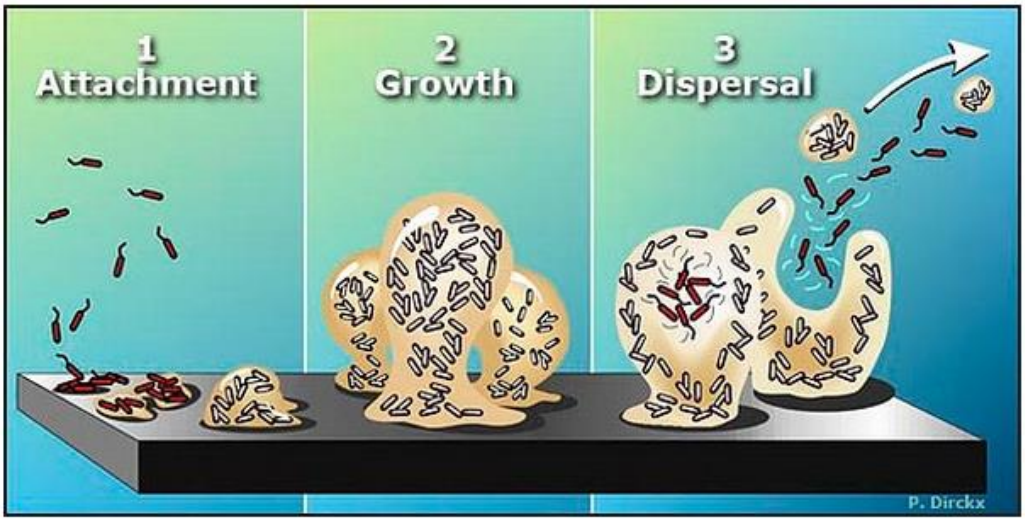

Figure 1.20 – The biofilm life cycle [63].

The life cycle is divided in 3 stages: 1) Free-floating, or planktonic, bacteria encounter a submerged surface and within minutes can become attached. They begin to produce slimy extracellular polymeric substances (EPS) and to colonize the surface. 2) EPS production allows the emerging biofilm community to develop a complex, three-dimensional structure that is influenced by a variety of environmental factors. Biofilm communities can develop within hours. 3) Biofilms can propagate through detachment of small or large clumps of cells, or by a type of "seeding dispersal" that releases individual cells. Either type of

detachment allows bacteria to attach to a surface or to a biofilm downstream of the original community [62].

The immobilized cells grow and reproduce, with the newly formed cells attaching to each other as well as to the surface. They also produce extracellular polymers, which extend from the cells to form a matrix of fibers [60].Within the biofilm, the microorganisms are organized into a real community which, through complex interactions with extracellular mediators and chemo-tactical, can work together and develop specific and complex functions, which are quite different from those made by individual bacterial colonies. Aggregates of more species (consortia) can form biofilms in which they are to create gradients chemical-physical phenomena of horizontal gene transfer and intensive inter-cellular communication; from this point of view, the biofilm are to all effects a micro-environment very competitive subjects at high pressures and evolutionary selection of organisms within them [63].

One benefit of this environment is the increased resistance to detergents and antibiotics, since the dense extracellular matrix and the outer layer of the cells protects the internal part of the community. In some cases the resistance to antibiotics can increase of 1000 times. The biofilm formation is also a benefit to the anaerobic bacteria.

Within the biofilm, the percentage by dry mass is constituted for approximately 10% from the cells and for about 90% from the matrix. This matrix is a complex set of components, which are called extracellular polymeric substances (EPS). The EPS plays many roles;in the Table 1.7 we can see these functions.

Table 1.7 – functions of extracellular polymeric substances in bacterial biofilms [63]

Function	Relevance for biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protoza	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Faciliates horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes

In nature, biofilms are produced by multi-species consortia. These micro-ecosystems are of great interest because the presence of multiple species within the same biofilm allows for greater adaptability to external conditions, due to the emergence of phenomena of cooperation between the different species. Moreover, the presence of different microorganisms has a potential of applicability greater than a mono-species biofilms.

The species that are integrated within the biofilm are more important, so as to understand the types of interactions that are established. There are several techniques used for the analysis of consortia present in biofilms. We distinguish cultivation techniques and crop. Some of the more applied methodologies are those of gene sequencing, PCR amplification

and cloning that allow to define which species are present, without the need to isolate them individually using pure cultures.

In the food industry the biofilm in general, can cause considerable technological problems such as: increased of resistance of a fluid, corrosion and resulting in a loss of product. The biofilm can be used for useful purposes. For example in many purifiers of water from sewers is included a treatment stage in which the water is passed through biofilm grown on filters, which extract and metabolize many organic compounds harmful.

2. AIM OF THE THESIS

The search for renewable sources has been increasing in recent years, mainly with use waste as substrates. The hydrogen in special is considered a vector energetic of future. The hydrogen can be produced from waste organics and when this production is performed by bacteria extremophiles, several advantages are present: yields of conversion of substrate to H_2 is closer to the yields theoretical maximum of 4 H_2/glucose, when working at high temperatures the risk of contamination is much smaller, low viscosity fluids and better mixing [35].

This thesis was aimed at studying the hydrogen production by extremophile bacteria, using food industry waste as substrates for fermentation.

The experimental work started from microcosm studies and ended with experimental tests in a 20 L innovative immobilized biomass bioreactor conducted in batch and in continuous. Main goals of the work were:

- ✓ choice of a buffer system capable of maintaining the pH for small scale fermentations (120 mL microcosms);
- ✓ optimization of the pH for the production of hydrogen;
- ✓ selection of the best strain Thermotoga for the production of H_2.
- ✓ minimization of the growth medium for the different substrate (milk whey and molasses), several studies were carried out to find a minimal medium without getting an excessive drop in the production of hydrogen.
- ✓ choice of the a biofilm carrier for the production of H_2 in a immobilized biomass bioreactor (biofilm reactor);
- ✓ development of a method for the quantitative analysis of immobilized biomass by its detachment from the media and determination;
- ✓ a preliminary kinetic study for different substrates: glucose, molasses and whey milk; study of the possible inhibition by substrate and product (H_2).
- ✓ a comparison of the perfomances and the kinetics of suspended and immobilized biomass;
- ✓ a study of possible inhibition of oxygen in suspended and immobilized biomass;
- ✓ feasibility tests of the process in a 20 L batch bioreactor conducted in batch and in continuous with suspended and immobilized biomass

3. MATERIALS AND METHODS

3.1 SUBSTRATE AND CHEMICALS

The molasses used in the experimental programme were, produced by the Trecasali sugar production plant of Eridania SpA (Parma, Italy), they contained 24.5% sucrose, 14.0% fructose, 9.5% glucose and 6.1% proteins. Its COD was equal to 0.85 g $g_{molasses}^{-1}$.

In the experiments with milk whey in order to maintain a constant composition during the entire study, a milk whey powder was used; it was produced by Tosi & G, Vimercate, Italy by a process of waste milk whey filtration and drying. It had the following composition: 83% lactose, 4% proteins, 1% fat, 4% moisture and 6% ash.

Its COD was equal to 1.11 g g milk whey $^{-1}$.

All the remaining chemicals were purchased from Sigma-Aldrich (Milan, Italy).

3.2 MICROORGANISMS

3.2.1 THERMOTOGA STRAINS

Four types of *Thermotoga* strains were used, which were compared for their fermentative hydrogen production abilities. They were *T. neapolitana* DSM 4359 (ATCC 49049), *T. maritima* DSM 3109, *T. naphtophila* DSM 13996 and *T. petrophila* DSM 13995. Microorganisms were obtained from DSMZ, the German Collection of Microorganisms and Cell Cultures.

Lyophilized cells were resuspended in slightly modified version of sterile ATCC 1977 media (*T. neapolitana*), DSM 1232 medium (*T. maritima*), and in DSM 913 medium (*T.petrophila* and *T. naphtophila*). The media chemical composition is listed below. In order to produce stocked cell suspensions for each strain, sterilized 116 ml-bottles with 40 ml fresh media were grown in anaerobic conditions using a 5% v/v inoculum. For the stock of cells using 1 ml aliquots of cells grown in this way for about 60 hours were added to 1 ml of fresh sterilized HEPES-buffered medium supplemented with 40 % v/v glycerol 5 % v/v DMSO. Stock cultures were conserved in 2 mL criovial tubes at -80ºC.

Stock cultures were usually used to inoculate sterile fresh media (*pre-inoculum*). The 60 hours-grown pre-inoculum was used either for criovials preparation or for 40 mL cultures inoculation (5 % v/v).

3.2.2 THERMOTOGA STRAINS AND STOCK PREPARATION

Chemical composition of growth media for four microorganisms is in Table 3.1, the modified version of the three media was supplemented with chloride salts replacing sulphate and sulfide salts. Moreover, glucose was used as main carbon source instead of starch in modified version of DSM 1232 (Table 3.2). When required, milk whey or sugar beet molasses replaced glucose at the concentration of 12.5 and 20 g/L, respectively, and HEPES was supplied at concentration of 23.8 g/L (100 mM).

Table 3.1 Composition of the culture media

Component	Strain		
	T. neapolitana (complete medium)	*T. maritima* (complete medium)	*T. naphtophila, T. petrophila* (complete medium)
NaCl (g L^{-1})	10	26	20
NH_4Cl (g L^{-1})	1	0.5	1
K_2HPO_4 (g L^{-1})	0.7	0.7	0.7
$MgCl_2$ $6H_2O$ (g L^{-1})	0.2	3	3
$CaCl_2$ $2H_2O$ (g L^{-1})	0.1	0.6	0.3
KCl (g L^{-1})	0.10	0	0.5
Microelements (DMS 141) (mL L^{-1})	10	10	10
Vitamins (DMS 141) (mL L^{-1})	10	10	10
Yeast extract[c] (YE) (g L^{-1})	2	2	2
Tryptic soy broth[d] (TSB) (g L^{-1})	2	2	0
Cysteine (g L^{-1})	1	0.5	1
Hepes (g L^{-1})	23.8	23.8	23.8
Resazurin (g L^{-1})	0.02	0.02	0.02
pH	8.5	7,5	7

The composition of vitamins and microelements solutions is shown in Tables 3.2 and 3.3.

Table 3.2 – Microelement solution DSM 141

Componets	mg/L
Nitrilotriacetic acid	1500
$MgSO_4 \cdot 7\ H_2O$	3000
$MnSO_4 \cdot H_2O$	500
NaCl	1000
$FeSO_4 \cdot 7H_2O$	100
$CoSO_4 \cdot 7H_2O$	180
$CaCl_2 \cdot 2\ H_2O$	100
$ZnSO_4 \cdot 7H_2O$	100
$CuSO_4 \cdot 5\ H_2O$	10
$KAl(SO_4)_2 \cdot 12\ H_2O$	20
H_3BO_3	10
$Na_2MoO_4 \cdot 2\ H_2O$	10
$NiCl_2 \cdot 6\ H_2O$	25
$Na_2SeO_3 \cdot 5\ H_2O$	0.3

Table 3.3 – Vitamin solution DSM 141

Components	mg/L
Biotine	2
Folic acid	2
Piridossine HCl	10
Tiamine HCl	5
Riboflavine	5
Nicotinic acid	5
D-Ca-pantotenate	5
Cobalamin (Vitamin B12)	0,1
p-aminobenzoic acid	5
Lipoic acid	5

The initial pH of the medium was adjusted to 8.5, 7.5 and 7.0, for *T. neapolitana*, *T. maritima*, *T. naphtophila*, *T. petrophila*, respectively, for each strain. The media were prepared aerobically, the 116 ml bottles were filled up with 40 ml of medium. Serum vials were then stoppered with butyl rubber stoppers, sealed and sparged with N_2 sterile by sterile needles for 5 minutes. Inoculated flasks were placed in an incubator shacking at 100 rpm and 77 °C. Appearance batch cultures in anaerobic state are shown in Figure 3.1.

During our study we demonstrated that the culture medium doesn't require sterilization, through various tests in the laboratory. We made DNA extraction to confirm that the only culture existing in the culture medium was *Thermotoga neapolitana*, the tests showed that in the culture medium were only *Thermotoga neapolitana*. Thus, media sterilization was not performed and the whole set of experiments was carried out in not sterile conditions if not otherwise specified. If sterilization occurred, bottles and media were previously autoclaved at 121°C for 20 minutes. Microelements, vitamins, $MgCl_2$, $CaCl_2$ and glucose filtered solutions were subsequently added to complete the media.

Fig. 3.1 – Batch cultures in anaerobic state ready to use. From the left, modATCC 1977 added respectively with glucose, milk whey and sugar beet molasses.

3.3 SUSPENDED CELLS TESTS

Experiments were conducted in triplicate. If not otherwise specified, initial and final samples for biomass and chemicals concentration quantification were taken while gases analyses were carried out once or twice a day for the entire period of the experiments. Incubation conditions are the same in all experiments, 100 rpm and 77 ° C.

3.3.1 SELECTION BUFFER

The list of buffers investigated for their capacity to maintain pH at physiological values during *T. neapolitana* growth, and, therefore, to preserve suitable hydrogen-producing conditions for this strain, is presented in Table 3.4.

Table 3.4 – Buffers tested in test of hydrogen production by *T. neapolitana* cultures on modATCC 1977 medium.

BUFFER	OPTIMAL pH	INITIAL Ph
Diacid/Monoacid Phosphate ($HPO_4^{-2}/H_2PO_4^-$)	6.8	8.5
Tris (hydroxymethyl) aminomethane (TRIS)	7.5 - 7.9 (8.5)	8.5
3-(N-morpholino) propanesulfonic acid (MOPS)	6.5 - 7.9 (7.2)	8.5
Piperazine-N, N'-bis(2-ethanesulfonic) acid (PIPES)	6.5 – 7.8 (6.8)	8.5
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	6.8 – 8.2 (7.5)	8.5

A batch process was considered completed when there was no further detectable hydrogen production; this usually occurred after 68-72 hours in modATCC 1977 defined media with glucose as main carbon source. Initial pH value of 8.5 was selected because it was the highest value of the optimal range that was found to result in optimal hydrogen productivity by *T. neapolitana* in batch cultures [51].

3.3.2 SELECTION OF THE THERMOTOGA STRAIN

Were studied 4 types of strain of Thermotoga: *T. neapolitana* DSM 4359 (ATCC 49049), *T. maritima* DSM 3109, *T. naphtophila* DSM 13996 and *T. petrophila* DSM 13995. All the assays were conducted in 116 mL glass vials sealed with rubber septa and incubated in a Dubnoff orbital shaker (General Laboratory Supply, Pasadena, USA) at 100 rpm, 77 °C and initial pH 8.5 [50] Before adding the inoculum, in each vial the growth medium was N_2-stripped until the resazurin colour disappeared. Each vial contained 38 mL of liquid medium and 2 mL of inoculum. These assays were monitored for 7 days, with daily measurements of headspace composition. Cells, sugars and organic acids were measured at the beginning and at the end of each assay. Before each headspace sampling, the pressure was regulated at 1 atm with a frictionless glass syringe.

3.3.3 MINIMIZING THE MEDIUM COST

The culture medium for *Thermotoga neapolitana* modATCC 1977 is composed of solutions of microelements, vitamins, reducing agent and other elements of high cost, which

makes the production of hydrogen in large scale not viable. In order to minimize the cost of hydrogen production from simplified medium for both molasses and milk whey, a study was conducted for the reduction of components of the reactants from the culture medium, without excessive losses in process performance. Main modATCC 1977 components were omitted step by step: microelements, vitamins, TSB, YE, cysteine and etc. More economic reducing agents were also tested, as metabisulfite (MBS). Macroelements (e.g. NaCl, NH_4Cl, K_2HPO_4) were considered essential for osmotic balance, and nitrogen and phosphate supply. KCl, $MgCl_2$ and $CaCl_2$ were included in microelements. Experiments were carried out for 1 week. The Tables 3.5 and 3.6 summarizes minimization strategy adopted in modATCC 1977 for molasses. The Tables 3.7 and 3.8 summarizes minimization strategy for milk whey.

Table 3.5 – Test of Minimization of the culture medium for *Thermotoga neapolitana* modATCC 1977 for molasse with cysteine as reducing agent

Test	HEPES g/L	NaCl g/L	H_2KPO_4 g/L	NH_4Cl g/L	TSB g/L	YE g/L	KCl g/L	$CaCl_2.2H_2O$ g/L	$MgCl.6H_2O$ g/L	Cys g/L	Vitamins mL/ L	µelement mL/L
1	23.8	10	0.7	1	2	2	0.1	0.1	0.2	1	10	10
2	23.8	10	0.7	1	2	0	0.1	0.1	0.2	1	10	10
3	23.8	10	0.7	1	0	2	0.1	0.1	0.2	1	10	10
4	23.8	10	0.7	1	2	2	0.1	0.1	0.2	1	10	0
5	23.8	10	0.7	1	2	2	0.1	0.1	0.2	1	0	10
6	23.8	10	0.7	1	2	2	0.1	0.1	0.2	0	10	10
7	23.8	10	0.7	1	0	0	0	0	0	1	0	0
8	23.8	10	0.7	5	0	0	0	0	0	1	0	0
9	23.8	10	0.7	1	0	0	0	0	0	0	0	0
10	23.8	10	0.7	1	0	0	0	0	0	0.5	0	0

Table 3.6 – Test of Minimization of the culture medium for *Thermotoga neapolitana* modATCC 1977 for molasse with MBS as reducing agent

Test	HEPES g/L	NaCl g/L	H_2KPO_4 g/L	NH_4Cl g/L	TSB g/L	YE g/L	KCl g/L	$CaCl_2.2H_2O$ g/L	$MgCl.6H_2O$ g/L	MBS g/L	Vitamins mL/ L	µelement mL/L
1	23.8	10	0.7	1	0	0	0	0	0	0.5	0	0
2	23.8	10	0.7	1	0	0	0	0	0	0.25	0	0
3	23.8	10	0.7	1	0	0	0	0	0	0.125	0	0
4	23.8	10	0.7	1	0	0	0	0	0	0.0625	0	0

Table 3.7 – Test of Minimization of the culture medium for *Thermotoga neapolitana* modATCC 1977 for milk whey with cysteine as reducing agent

Test	HEPES g/L	NaCl g/L	H_2KPO_4 g/L	NH_4Cl g/L	TSB g/L	YE g/L	KCl g/L	$CaCl_2.2H_2O$ g/L	$MgCl.6H_2O$ g/L	Cys g/L	Vitamins mL/ L	µelement mL/L
1	23.8	10	0.7	1	2	2	0.1	0.1	0.2	1	10	10
2	23.8	10	0.7	1	2	2	0.1	0.1	0.2	1	10	0
3	23.8	10	0.7	1	0	2	0.1	0.1	0.2	1	10	10
4	23.8	10	0.7	1	2	2	0.1	0.1	0.2	1	0	10
5	23.8	10	0.7	1	2	0	0.1	0.1	0.2	1	10	10
6	23.8	10	0.7	1	2	0	0.1	0.1	0.2	1	0	10
7	23.8	10	0.7	1	0	0	0.1	0.1	0.2	1	0	10
8	23.8	10	0.7	5	2	0	0	0	0	1	0	0
9	23.8	10	0.7	1	2	0	0.1	0.1	0.2	0	0	0
10	23.8	10	0.7	1	2	0	0.1	0.1	0.2	1	0	0
11	23.8	10	0.7	1	0	0	0	0	0	1	10	0
12	23.8	10	0.7	1	0	2	0	0	0	1	0	0
13	23.8	10	0.7	1	2	0	0	0	0	1	0	0
14	23.8	10	0.7	1	1	1	0.1	0.1	0.2	1	0	0
15	23.8	10	0.7	1	2	0	0.1	0.1	0.2	1	0	0
16	23.8	10	0.7	1	0	2	0.1	0.1	0.2	1	0	0

Table 3.8 – Test of Minimization of the culture medium for *Thermotoga neapolitana* modATCC 1977 for milk whey with MBS as reducing agent

Test	**HEPES g/L**	**NaCl g/L**	**H_2KPO_4 g/L**	**NH_4Cl g/L**	**TSB g/L**	**YE g/L**	**KCl g/L**	**$CaCl_2.2H_2O$ g/L**	**$MgCl.6H_2O$ g/L**	**MBS g/L**	**Vitamins mL/ L**	**µelement mL/L**
1	23.8	10	0.7	1	2	0	0	0	0	0.5	0	0
2	23.8	10	0.7	1	2	0	0	0	0	0.25	0	0
3	23.8	10	0.7	1	2	0	0	0	0	0.125	0	0
4	23.8	10	0.7	1	2	0	0	0	0	0.0625	0	0

3.4 ATTACHED CELLS TESTS

In immobilized cells experiments, 40 mL of porous carrier and 40 mL of liquid medium (enough to cover the carrier) were added into serum bottles. A 5 % v/v inoculum was performed.

Media preparation was executed as previously described. The whole set of experiments with immobilized cells was carried out in not sterile conditions. Hydrogen production by immobilized cells was divided into two steps. The first step consisted in the

inoculation of the batch cultures and biofilm formation on solid carrier. After 3 days, carriers were washed twice with 40 ml of saline solution. Initial biomass was measured by sampling 5 solid carrier; biomass quantification method is described below. In the second step, fresh medium was added to bottles and hydrogen production was followed for 3-5 hours after incubation. Experiments were carried out in triplicate. Gas quantification was performed every hour. Carbohydrates composition was analysed by sampling each batch culture at the beginning and at the end of the test, and every hour in one batch culture for each triplicate. Final biomass was also measured. In Figure 3.2 report an illustration of the tests with immobilized biomass

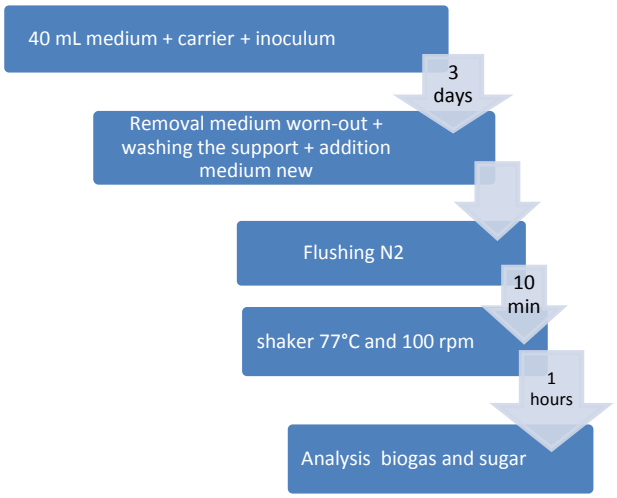

Figure 3.2 – Illustration of tests with immobilized biomass.

3.4.1 POROUS CARRIERS FOR BIOFILM FORMATION

For the tests with biofilm were studied four biomass carrier types, in Table 3.9 are indicated the characteristics of the carrier used for this study. In the Figure 3.3 below the 4 carrier used.

Table 3.9 – Porous carrier characteristics

Carrier	Porosity (%)	Density (Kg/L)	Material
Biomax®	67	0.777	Ceramic
Glaxstone	75	0.593	Syntherized glass
Siporax® cylinders	82	0.570	Syntherized glass
Siporax® mini	70	0.535	Syntherized glass

Figure 3.3 – Porous carrier, (a) Biomax®; (b) glaxstone; (c) Siporax® cylinders; (d) Siporax® mini.

3.5 KINETIC STUDIES

3 types of kinetic studies were performed during the PhD, in order to obtain parameters and evaluate the production of hydrogen. The method for biomass growth is the same as described above.

3.5.1 INHIBITION OF OXYGEN

Thermotoga strains are anaerobic, which means they are intolerant to the presence of oxygen. Due to this fact it was decided to conduct a study to assess if the strain *Thermotoga neapolitana* is able to tolerate small concentrations of oxygen. The study was conducted only for milk whey in conditions of suspended and immobilized biomass.

After the growth of the biomass, was added oxygen, the concentration of O_2 for the test with suspended cells were: 0; 0.1; 0.5 and 1 %. For the attached cell were: 0; 0.01; 0.05; 0.10; 0.25; 0.5 and 1 % of oxygen in the head space.

3.5.2 INHIBITION OF HYDROGEN

To assess whether during the production of hydrogen with *Thermotoga neapolitana* product, we performed a study with all substrates used during the PhD in the conditions of suspended and immobilized biomass. The conducted biomass growth for the test is the same as described above.

For the test with suspended biomass, the first step is the growth of biomass and total consumption of sugars culture medium. After total consumption of the sugars the second step is the addition of 5 mL of medium concentrated to leave the medium in the desired sugar concentration and the addition of H_2 in the head space.

For the immobilized biomass the first step consisted in the inoculation of the batch cultures and biofilm formation on solid carrier. After 64 hours, carrier were washed twice with 40 ml of saline solution. In the second step, fresh medium was added to bottles and

hydrogen production was followed for 3-5 hours after incubation. In the Table 3.10 there is a schema of Tests.

Table 3.10 – Schema of the test Hydrogen Inhibition

Substrate	Culture Medium	Biomass	H_2 %
GLUCOSE 7.5 g/L	Medium complete	Immobilized	0.73 13 25 37 47 56
GLUCOSE 7.5 g/L	Medium complete	Suspended	0.35 10 18 30 40 50
MOLASSES 20 g/L	Medium Minimum	Immobilized	2 10 18 29 37
MOLASSES 20 g/L	Medium Minimum	Suspended	0 6 16 29 45 58
MILK WHEY 12.5 g/L	Medium Minimum	Immobilized	0.73 13 25 37 56
MILK WHEY 12.5 g/L	Medium Minimum	Suspended	0.39 13 25 38 51

3.5.3 INHIBITION OF SUBSTRATE

The tests Inhibition of substrate were realized with all substrate used in this PhD and 2 conditions of biomass.

Kinetic parameters calculation was executed on Microsoft Excel 2003®. The modelling of experimental data was performed by applying the Haldane model:

$$q_{H2} = \frac{q_{\max,H2} \cdot S}{K_S + S + S^2/K_I}$$

Hydrogen specific production rate (q_{H2}) was assumed proportional to specific substrate consumption rate. S is substrate concentration, K_S is the substrate constant, K_I is the substrate inhibition constant and q_{max}, H_2 is the maximum specific hydrogen production rate. A first set of possible values was given to the constants in the software in order to calculate for each tested substrate the specific rate of hydrogen production. By using the calculated values, the square standard deviation was evaluated for each condition and, then, this deviation was minimized by the software changing the initial values through a trial by error method.

Condition of tests the inhibition of substrate in the Table 3.11.

Table 3.11 – Schema Inhibition substrate.

Substrate	Culture Medium	Biomass	Sugars g/L
GLUCOSE	Medium complete	Immobilized	0.25 0.5 0.7 0.8 17 48 95
GLUCOSE	Medium complete	Suspended	0.36 0.65 0.89 1.5 6 18 36 51
MOLASSES	Medium Minimum	Immobilized	0.27 0.6 0.7 1.5 5 7 13 33
MOLASSES	Medium Minimum	Suspended	0.10 0.3 0.4 2.5 6 12 31
MILK WHEY	Medium Minimum	Immobilized	0.32 0.45 0.6 0.8

			3.6 17 36 72
MILK WHEY	**Medium Minimum**	**Suspended**	**0.42** **0.6** **0.7** **0.9** **3** **12** **32** **60**

3.6TEST BIOREACTOR

Has been realized a jacketed reactor, in stainless steel, the volume of 19 L (T = 0.23 m, H / T = 2), equipped with: 2 glass windows; 2 PBT impellers, pumping down; sparger for the distribution of gas (nitrogen) supplied from the bottom; pH control (ON / OFF); flow meter of biogas produced; preparation for connection to the circuit of biogas purification.

The insertion in the reactor of a draft-tube interior, combined with the use of 2 PBT impellers that pump down, allows obtaining a slight vacuum in the head space of the draft-tube, and then a movement of the biogas in the circuit of hydrogen purification, avoiding the use of a fan (Figure 3.4).

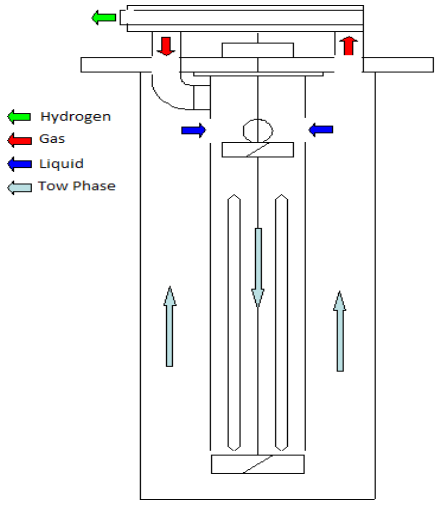

Figure 3.4 - Description of the draft tube.

This reactor has a major innovation, for the tests with immobilized biomass, which is the space dedicated to bags in steel, where the carrier are placed for biofilm growth. Biomax® carrier for biofilm Thermotoga are contained within bags made of stainless steel

mesh arranged radially, between the wall of the reactor and the tube suction. This solution allows: devote most of the volume of the bioreactor to the carrier for the biofilm, allow a good circulation of the gas produced. In Figure 3.5 we can see a photo of the bags inside the reactor.

Figure 3.5 - The bags with carrier.

In the experiments with the bioreactor were used the means described above and in conditions not sterilized. For the experiments in the bioreactor first added was the culture medium no sterile 14L, then closing reactor and start the fluxing medium with N_2 to remove oxygen in the medium. After the medium reach a state of anaerobic is addiction the inoculum 1% v / v.

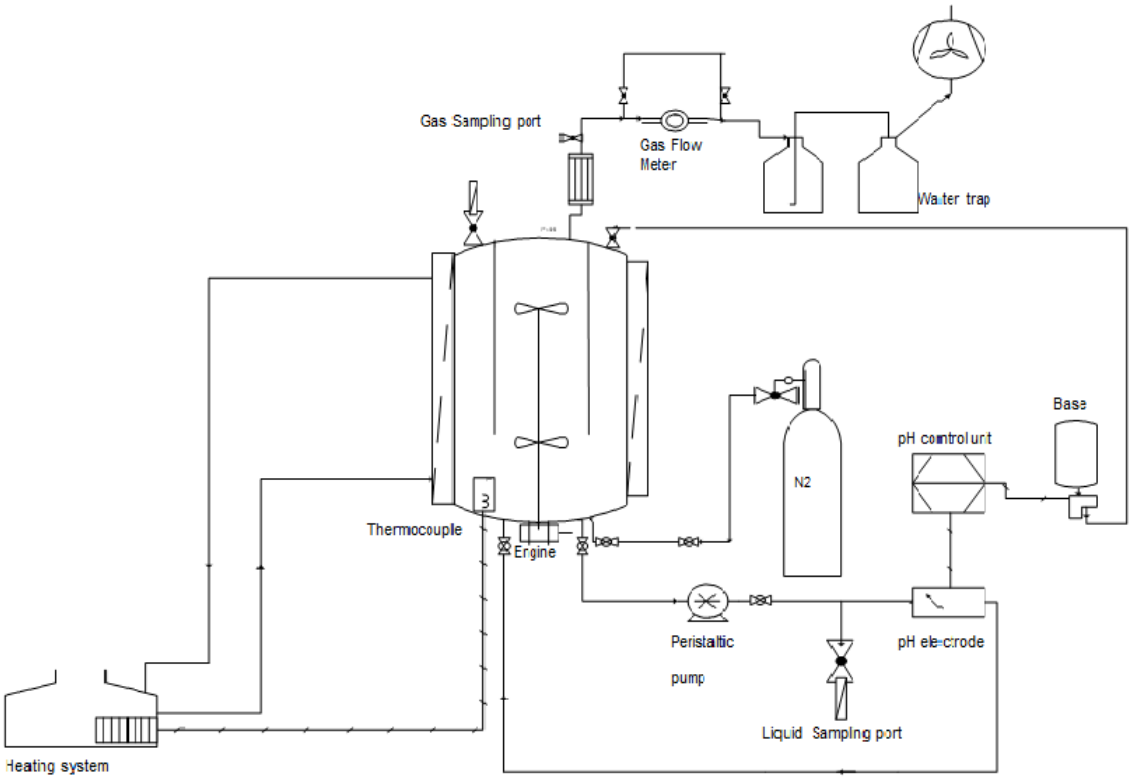

Figure 3.6 - Diagram of the SPCSTR Hydrogen bioreactor**.**

3.7 - ANALYTICAL METHODS

3.7.1 - HIGHLIGHT BIOMASS

The concentration of attached cells was determined according to the following procedure: replacement of the vial liquid phase with 40mL of de-ionized (DI) water, to remove the suspended cells; 18 h shaking (100 rpm, 77◦C) to obtain a first biofilm detachment and to allow the residual growth medium entrapped in the carriers porosity to dilute in the added DI water; vial centrifugation (10min, 8000 rpm) and supernatant removal, to attain a first removal of the residual growth medium; re-addition of 40mL of DI water; 1 h shaking (100 rpm, 77◦C); 2nd centrifugation step(10min, 8000 rpm) followed by supernatant removal; addition of 30mL of NaOH 1mol L−1; 1 h shaking (100 rpm, 77◦C), to allow the NaOH to react both with the residual biofilm cells and with the cells detached in the previous shaking steps. At this point, the traditional Lowry method was used [66].

3.7.2 - TOTAL PROTEIN

The quantification of total protein was made using the method of Lowry [66]. Lowry method was employed for protein content of suspended cells lysates. Solutions employed are listed in Table 3.12.

Table 3.12 – Solutions employed in Lowry protein quantification method

Solution A	A 2% of Na_2CO_3in NaOH 0.1 N
Solution B1	B1 1% $CuSO_4 \cdot 5\ H_2O$ in distilled water
Solution B2	2% Na-tartrate in distilled water
Solution C	Solutions A: B1: B2 in a 100:1:1 ratio
Solution E	Folin-Ciocalteau phenol reagent 2N dilution in distilled water

A calibration curve was obtained by using bovine seric albumin (BSA) samples at different concentrations. 1 mg/ml BSA stock solutions were conserved in aliquots of 1 ml at -20°C until use. BSA standard samples for calibration curve calculation were prepared in duplicate. Concentrations used for the calibration curve are reported in Table 3.13.

Table 3.13 – BSA standard concentrations.

H_2O (µL)	BSA (µL)	[BSA] (mg/L)
500	0.0	0
468	32.5	10
435	65	20
402.5	97.5	30
337.5	162.5	50

The first stage for the analyses the total protein is preparation of the samples: 1 mL-samples of cell cultures are centrifuged at 13000 rpm for 10 minutes, pellet is washed twice with an equal volume of saline solution, centrifuged at 13000 rpm for 10 minutes and then resuspended in saline solution. Finally, 50-300 µL of cell suspensions are added to 100 µL of NaOH 0.5 N in order to obtain a total volume of 500 µL with water addition. 200 µL of Thermotoga cultures grown for 48-72 hours were usually employed. The stage is not necessary for test with immobilizes cells.

The Lowry method procedure that has been performed in protein quantification is explained below. The only protein samples were boiled in distilled water for 1 minute and were readily transferred in ice. 2.5 ml of solution C was added to the standard and protein samples that were immediately mixed by vortexing. The standard and protein samples were incubated for 10 minutes at room temperature. 250 µL of solution E was subsequently added to the standard and protein samples that were mixed immediately by vortexing. All the samples were incubated for 30 minutes at room temperature. The samples were finally read at the spectrophotometer at a wavelength of 540 nm. The protein concentrations were obtained through interpolation of the absorbance data of the samples with the calibration curve and also by considering dilution or concentration factors.

3.7.3 – BIOGAS

Biogas produced by batch cultures was periodically measured. Hydrogen and carbon dioxide composition in the head space was analyzed by using an Agilent 3000 micro gas chromatograph (µ-GC)coupled with a Thermal Conductivity Detector (TCD). The instrumental method utilized is summarized in Table 3.14.

Tab. 3.14 – µ-GC analysis parameters

Pre-column temperature	90°C
Column temperature	60°C
Pressure	25 psi
Gas carrier	Nitrogen
Time of analysis	44s

Hydrogen and carbon dioxide calibration was carried out in triplicate with 116 sealed serum bottles. Hydrogen concentrations employed were 1, 10, 20, 30 and 40%. Carbon dioxide concentrations used were 1, 10, 50 and 100%. Sampling was executed from batch cultures at 77°C by injection through the butyl rubber stopper of a needle directly connected to the µ-GC. Sampled volume of headspace was 2 ml. Biogas volume was previously measured with 5-100 ml glass syringes. 2ml of biogas were put back into the head space in order to report atmospheric pressure values into the head space after every µ-GC analysis. Hydrogen and carbon dioxide productions were subsequently calculated considering both biogas volumes and headspace gas concentrations. The amounts of gases that are assumed to be dissolved in liquid phase at 77°C was taken into account. Biogas produced by bioreactor was periodically measured. Hydrogen and carbon dioxide composition production was analyzed by using a gas chromatograph (GC) coupled with a Thermal Conductivity Detector (TCD). The instrumental method utilized is summarized in Table 3.15.

Tab. 3.15 – GC analysis parameters

Column temperature	150°C
Pressure	7 psi
Gas carrier	Nitrogen
Time of analysis	3 min

Hydrogen and carbon dioxide calibration was carried out in triplicate with 116 sealed serum bottles. Hydrogen concentrations employed were 1, 10, 20, 30, 40 and 70%. Carbon dioxide concentrations used were 1, 5, 10, 20 and 30%. Sampling was executed from batch cultures at 77°C by injection splitless through the glass syringe in the GC. Sampled volume of headspace was 500 µL. Hydrogen and carbon dioxide productions were subsequently calculated considering both biogas volumes and headspace gas concentrations. The amounts of gases that are assumed to be dissolved in liquid phase at 77°C were taken into account.

3.7.4 – SUGARS AND VOLATILE FATTY ACIDS

The concentrations of sugars and Volatile Fatty Acids (VFAs) present in organic substrate and media were evaluated through high pressure liquid chromatography (HPLC). 1 ml-samples from each culture were collected and centrifuged for 10 minutes at 13000 rpm. Supernatant was filtered through 0.22 μm filters. HPLC method employed is summarized in Table 3.16 HPLC loop was washed with 10 μl of sample before sample injection.

Table 3.16 - Description HPLC

Column and pre column	Coregel-87H Transgenomic
Detector	Shimadzu Refraction Index detector (RID) 10A
Temperature of column	40°C
Mobile phase	0.01N H_2SO_4
Mobile phase flux	0.6 mL/min
pressure	54-60 bar

4. RESULTS AND DISCUSSION

4.1 FEASIBILITY STUDY OF HYDROGEN PRODUCTION FROM SELECTED SUBSTRATES

Through studies conducted by HERA, it was found that molasses and milk whey are the most abundant and problematic waste in the Emilia Romagna region. As shown in Table 4.1 molasses and milk whey are rich in sugars, thus they could be suitable growth substrate for fermentations using Thermotogas. For these reasons, molasses and milk whey have been chosen as substrates for our study.

Table 4.1 – Characteristics of molasses and milk whey.

	Sucrose %	Lactose %	Glucose %	Fructose %	Protein %	COD gg^{-1}
Molasses	**24.5**	**0**	**6.1**	**9.5**	**6.1**	**0.85**
Milk Whey	**0**	**83**	**0**	**0**	**1**	**1.11**

However both the matrices are complex, residuals of industrial processes that do not facilitate this conversion. For this reason additional tests were performed with a simple substrate, namely glucose. The tests were conducted using *Thermotoga neapolitana* and suspended biomass. The culture medium used in the tests was the one described in Table 3.1 in Materials and Methods. In the Figure 4.1 the results of the tests are reported.

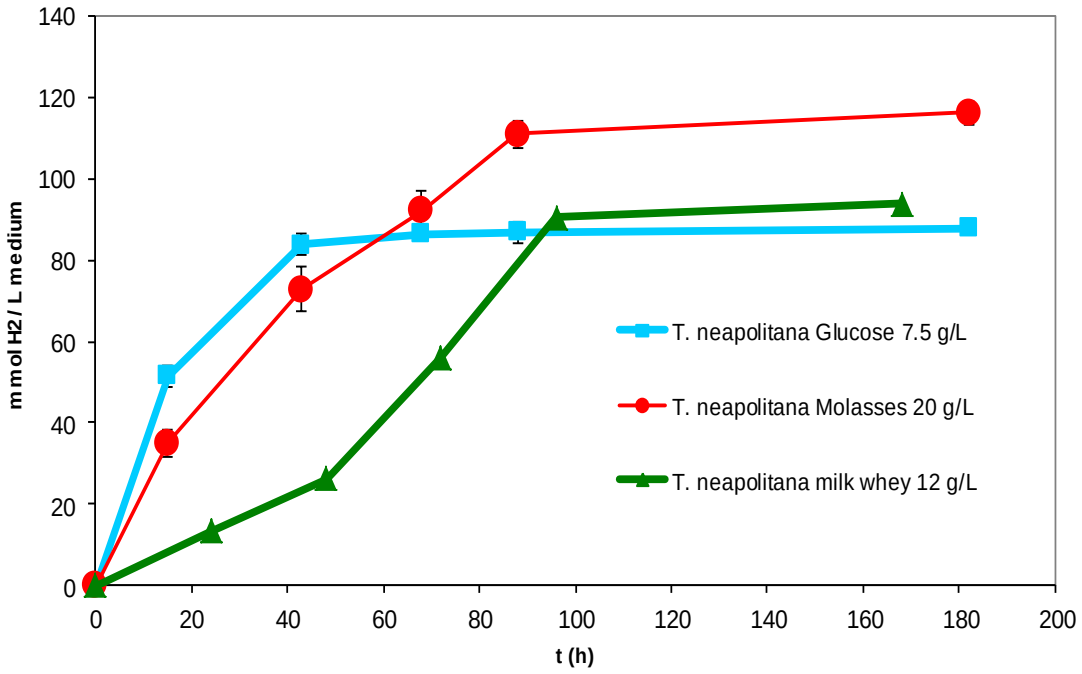

Figure 4.1 – Hydrogen production from Glucose, Molasses and Milk whey.

In Figure 4.1 we can see that *Thermotoga neapolitana* is able to convert molasses and whey in H_2 and, very important, that the results of production are higher than those obtained with glucose. This result is due to the fact that because they are complex matrices coming from industrial processes, they are rich in micro nutrients which favor the growth of *Thermotoga neapolitana* and other substrates that can be converted in H_2.

4.2SELECTION OF A BUFFER SYSTEM FOR THE PRODUCTION OF H_2

The pH control during the biological production of H_2 from Thermotoga is very important, the organic acids produced tend to lower the pH, the production of H_2 from Thermotoga strains is significantly slower at pH <5. For the batch at small scale (120 mL), some tests were performed to select a buffer system capable of controlling pH during the production of H_2.

Were evaluated 5 buffers, the selection of these buffers is based on data from the literature as shown in Table 1.4. The tests for the choice of the buffer were realized using *T. neapolitana* with different buffers (Table 3.4).

In Figure 4.2 and 4.3 we report the results for the tests with the 5 buffers.

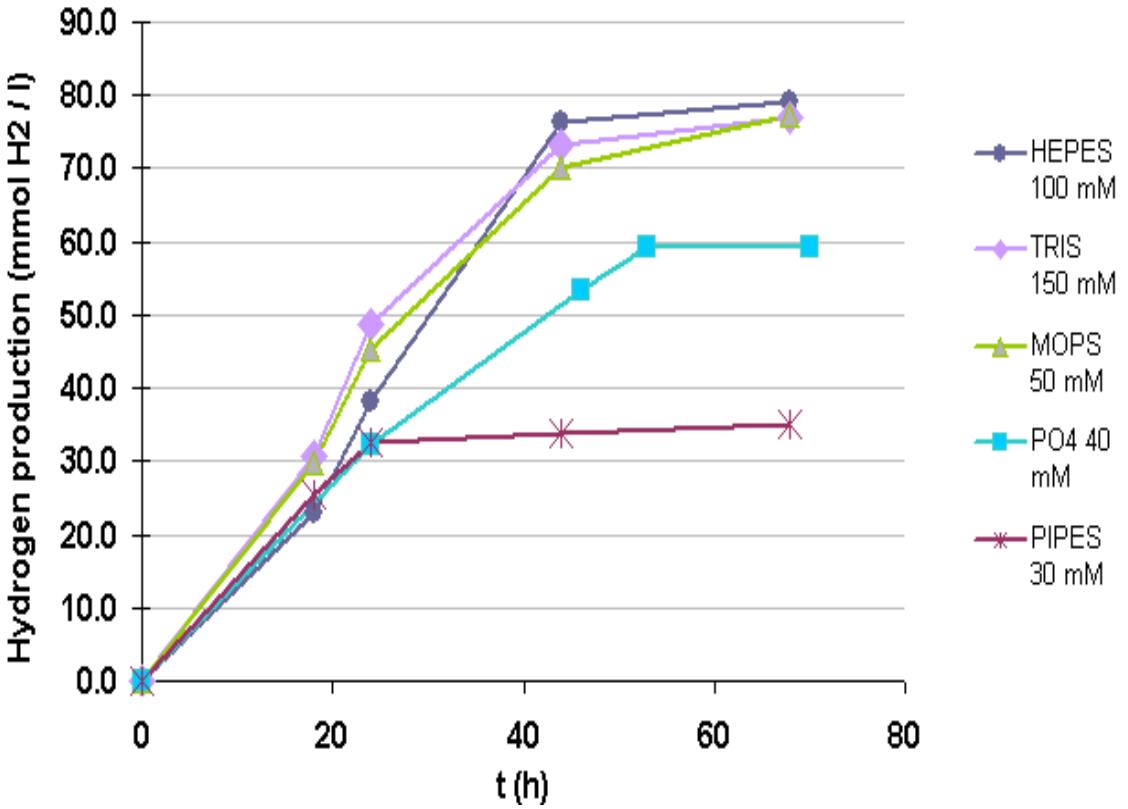

Figure 4.2 - Time course of hydrogen production by *T. Neapolitana*

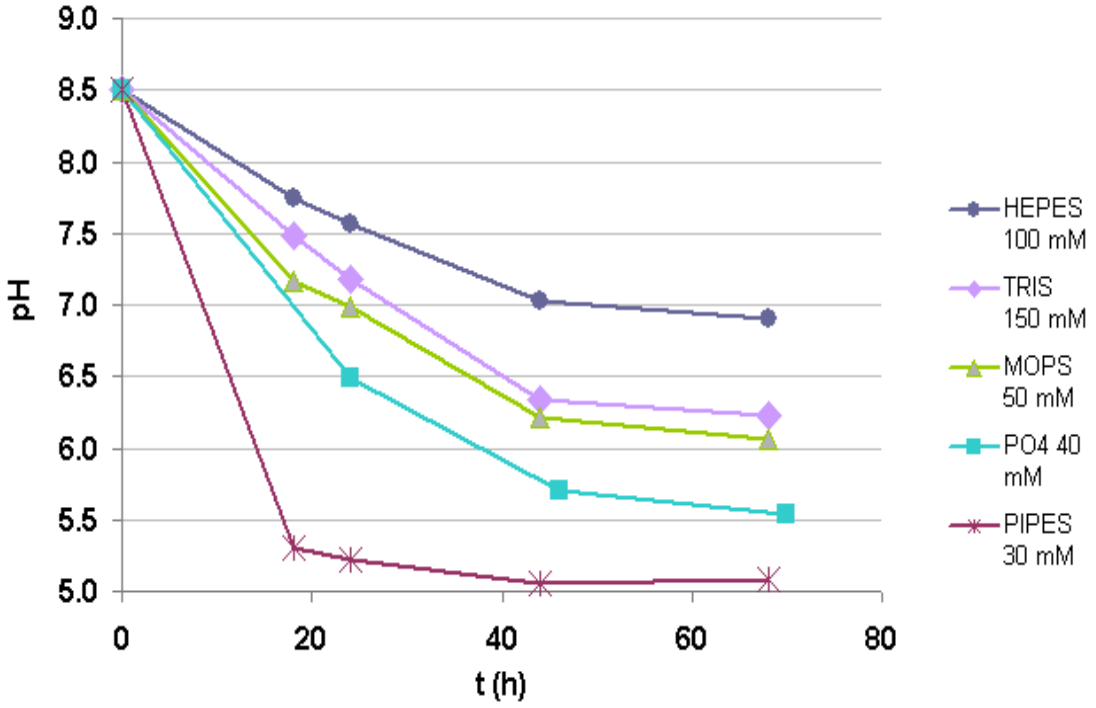

Figure 4.3- Time course of pH values.

In Figure 4.2 we can see that Hepes 100 mM, Tris 15 mM and MOPs 50 mM presented the best productions of H_2 in comparison with the other 2 buffers. In relation to the control of pH we can see in Figure 4.3 that Hepes 100 mM showed better control of pH. Using HEPES as the buffer, after 70 h of fermentation the H_2 productivity was 79 mmol/L at pH 7.0; it should be, noted that the initial pH was 8.5.

About the results shown in Figure 4.2 and 4.3 we can say that, organic buffers (except for PIPES) were more efficient than inorganic buffer in terms of buffering capacities and hydrogen production by *T. neapolitana* batch cultures.

After verifying that HEPES buffer was the most appropriate for our study, a test was performed with five different concentrations of HEPES, to evaluate the behavior of productivity with variation of buffer concentration. The concentrations studied of HEPES were: 50.100, 150, 200, 500 mM. In Figures 4.4 and 4.5 we can see the results of productivity and control of pH versus time respectively.

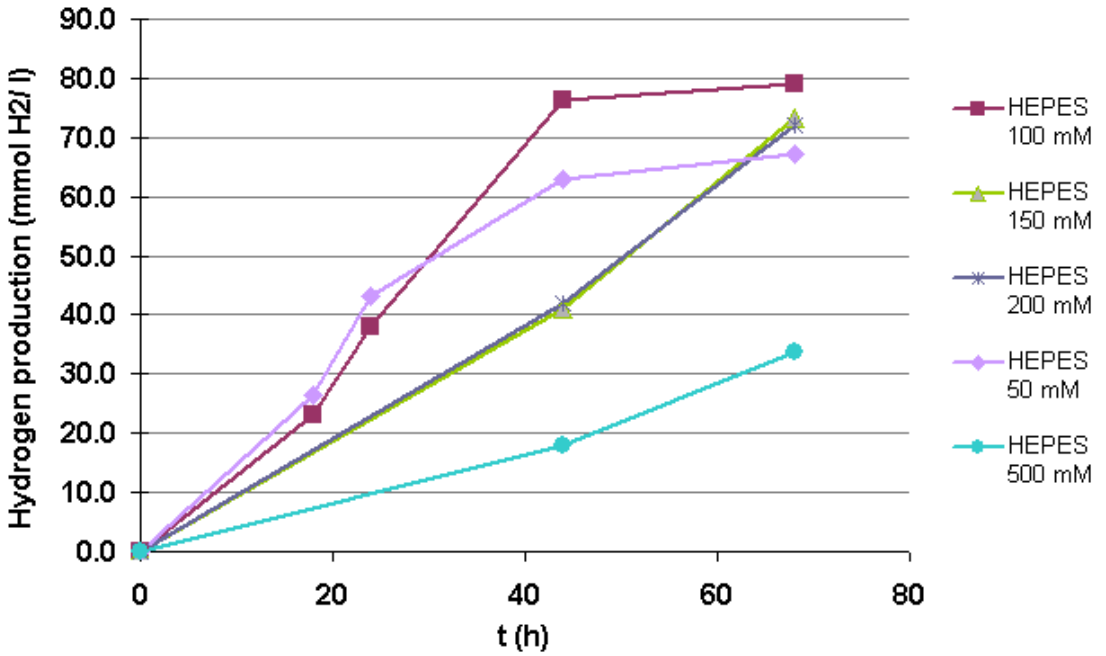

Figure 4.4 - Time course of hydrogen production by *T. neapolitana* for different concentration HEPES.

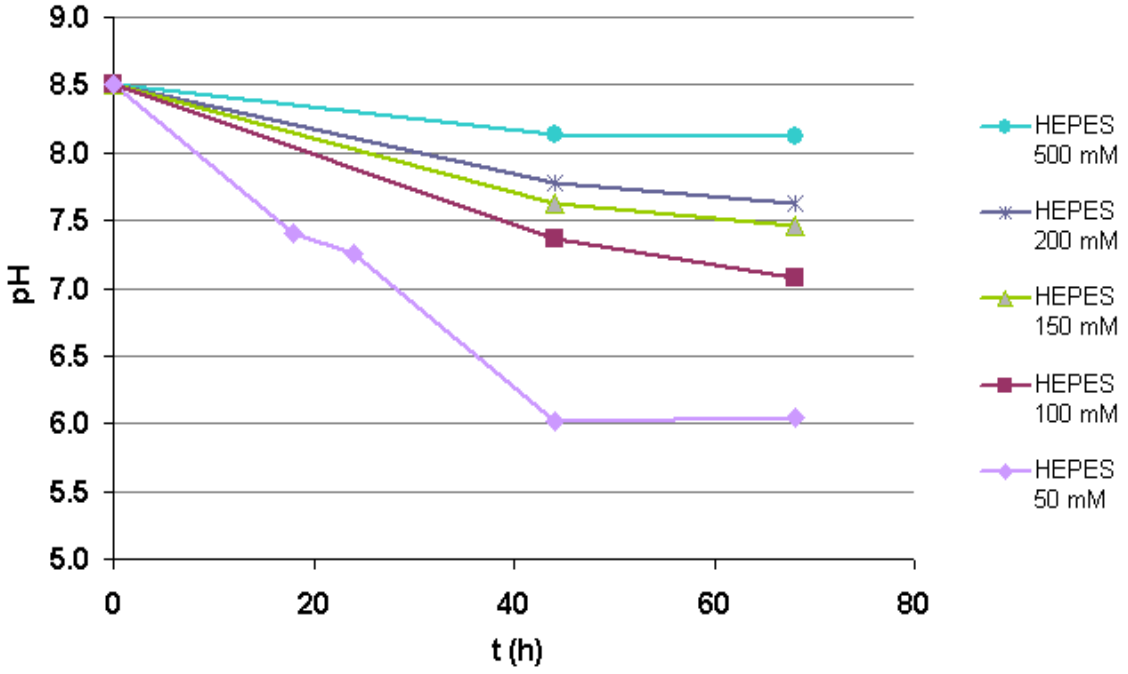

Figure 4.5 - Time course of pH values for different concentration HEPES.

The tests showed that at the concentrations of HEPES 50-200 mM the H_2 productivity after 60 hours is very similar about 80 mmol H_2/L. Instead using 500 mM HEPES there was a great reduction in productivity about 50% less than the one obtained with other buffers. The high buffer concentration inhibited the production of hydrogen, in Figure 4.5 we can see that

with HEPES to 500 mM there was a good pH control, due to high buffer concentration and low production of H_2, which means lower production of acids. We can conclude through Figures 4.4 and 4.5 which 100 mM of HEPES is the optimal concentration for the studies, 100 mM HEPES obtained the best productivity and control of pH.

4. 3 SELECTING THE THERMOTOGA STRAIN

Based on published data (Table 1.3), the bacteria of the genus Thermotoga are shown as the most interesting for the production of H_2. Four Thermotoga strains were chosen for our study and tests were performed to evaluate the 4 strains for production of H_2.

Strains studied were *T. neapolitana, T. maritima, T. naphtophila* and *T. petrophila*. The tests were performed with glucose, molasses and milk whey as a substrate and suspended and immobilized biomass condition, the support used for immobilization was *Biomax*®.

In Table 4.2 there is a summary of the selection tests for the Thermotoga strain.

Table 4.2 – Selection of the Thermotoga strain test summary.

Strain	Substrate	Biomass
T. naphtophila	Glucose, molasses and milk whey	Suspended and immobilized
T. neapolitana	Glucose, molasses and milk whey	Suspended and immobilized
T. maritima	Glucose, molasses and milk whey	Suspended and immobilized
T. petrophila	Glucose, molasses and milk whey	Suspended and immobilized

Figure 4.6 report the results with the four strains in terms of volumetric production of hydrogen for the three substrates. In Figure 4.7 we have volumetric CO_2 production. Glucose and molasses were tested at the same time, for milk whey it was not possible to monitor the production together with the other substrate.

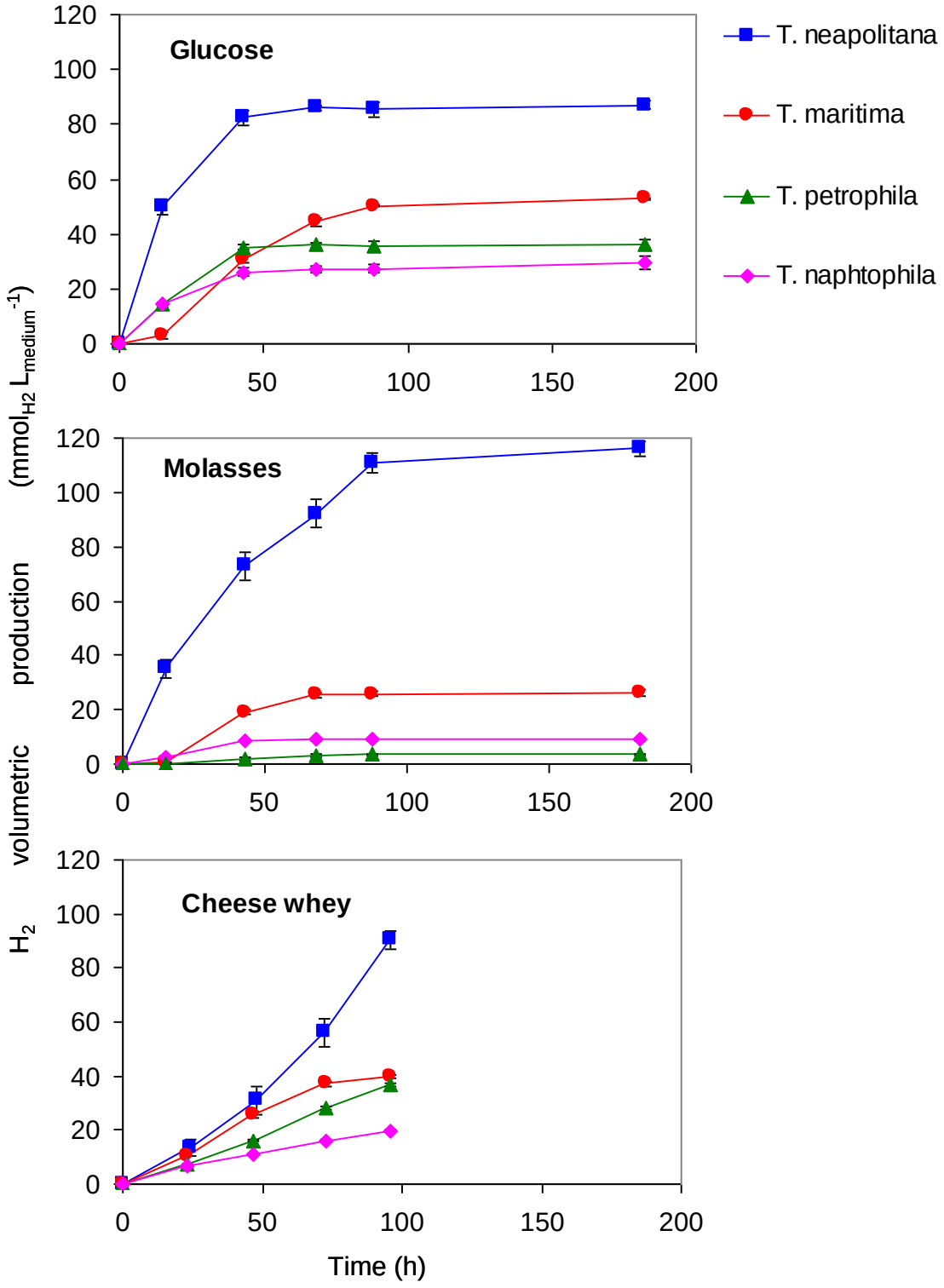

Figure 4.6 - Time profiles of H_2 volumetric production obtained with the suspended cells of the 4 *Thermotoga* strains. Average data in each triplicate series ± standard deviations.

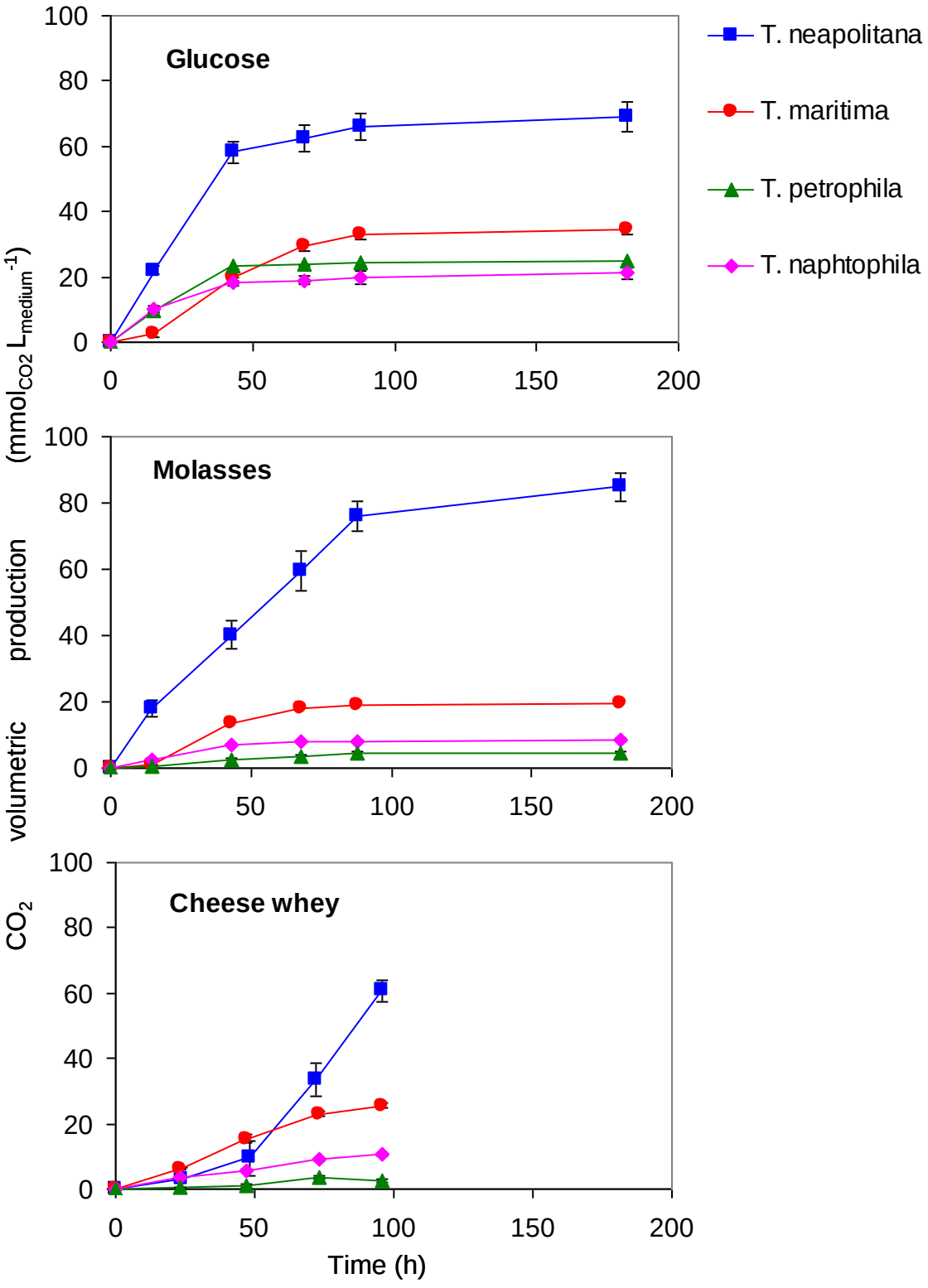

Figure 4.7 Time profiles of volumetric CO_2 production obtained with the suspended cells of the 4 *Thermotoga* strains. Average data in each triplicate series ± standard deviations.

The main results are shown in Figure 3(A), 3(B), 3(C) and 3(D) in terms of average H_2 rate during the initial 2 days, as well as $Y_{H2/S}$, $Y_{acetate/S}$ and $\eta_{E/S}$ during the entire 7-day test.

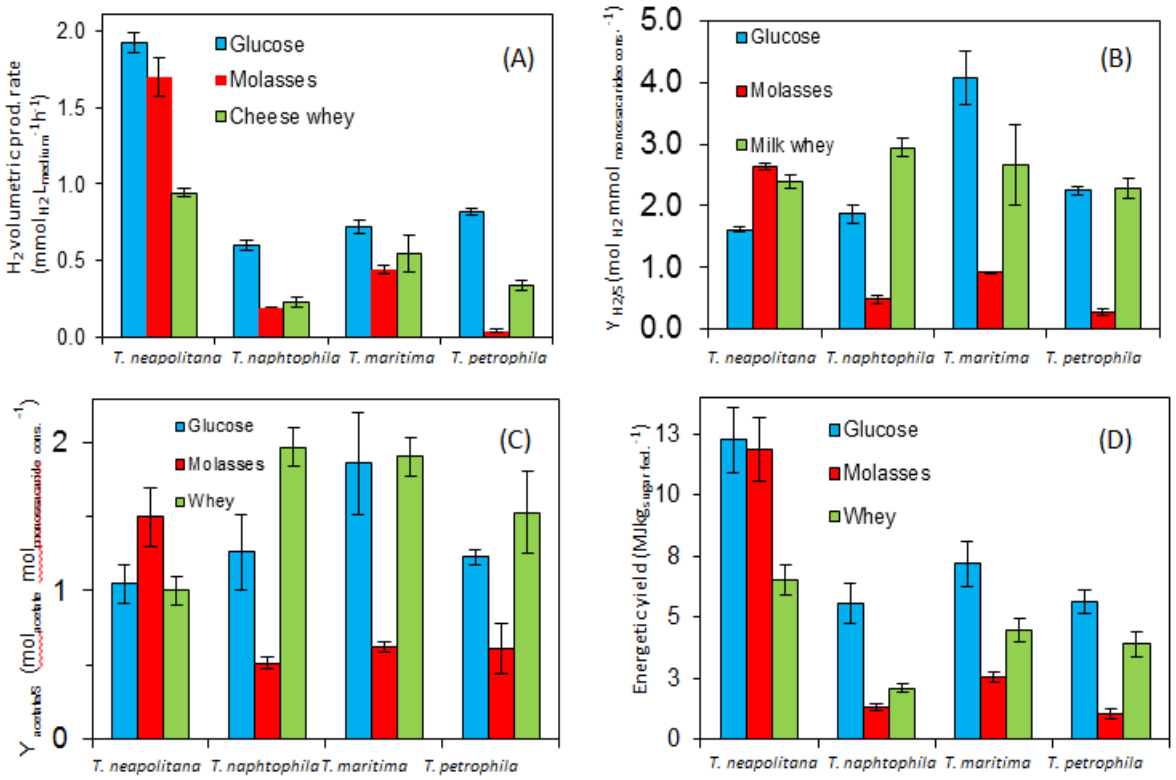

Figure 4.6 - H_2 production performances obtained with four Thermotoga strains suspended tests.

In terms of H_2 production rate, *T. neapolitana* was markedly superior to the other three strains with all three substrates tested, in the suspended-cell. The other three strains formed a homogeneous cluster only in the case of H_2 production from milk whey under suspended-cell conditions. In terms of $Y_{H2/S}$, with molasses, the best strain was *T. neapolitana*; with glucose, *T. maritime* performed better, and with milk whey no significant differences were detected among the four strains. Notwithstanding this, *T. neapolitana* was the best in terms of $\eta_{E/S}$ with all three substrates, thanks to the higher substrate conversions attained with this strain. As for the biomass/substrate yield, on both glucose and molasses, the highest values were obtained with (60–80$mg_{protein}$ $g^{-1}_{sugar\ consumed}$), whereas on cheese whey the highest growth was observed for *T. naphtophila* (150 $mg_{protein}$ $g^{-1}_{sugarconsumed}$) Table 4.3.

Table 4.3 – Date of biomass/substrate for selection Thermotoga strain in suspended cell tests.

Strains	**Biomass/substrate ($mg_{protein}$ g^{-1} sugar consumed)**		
	Glucose	Molasses	Milk whey
T. neapolitana	59	80	60
T. naphtophila	40	20	150
T. maritima	30	10	60
T. petrophila	50	10	20

The better performances of *T. neapolitana* might be ascribed to its tolerance to higher concentrations of dissolved H_2 and O_2, in comparison with the other strains [27, 49].

The $Y_{H2/S}$ attained with *T. maritima* is equal to the theoretical maximum value of 4mol H_2 $mol^{-1}_{monosaccharide\ consumed}$, as previously described by Schröder et al [64].

Conversely, the $Y_{H2/S}$ attained with *T. naphtophila* and *T. petrophila* were 45–50% lower than the corresponding values reported by Takahata et al. (about $4mol_{H2}$ $mol^{-1}_{monosaccharide\ consumed}$, for both *T. naphtophila* and *T. petrophila*) [36].

Was also performed the carbon balance in this case only for *T. neapolitana*, strain that showed better performance in suspended cells conditions, with the 3 substrates. The results of the carbon balance analysis are shown in detail in Figure 4.8. The fraction of consumed organic carbon found in the process products (CO_2 + acetate + lactate + cells) was equal to 105% ± 11% with glucose, 97% ± 13% with molasses, and 95% ± 12% with milk whey. These results indicate that other possible process products were negligible in comparison with those listed.

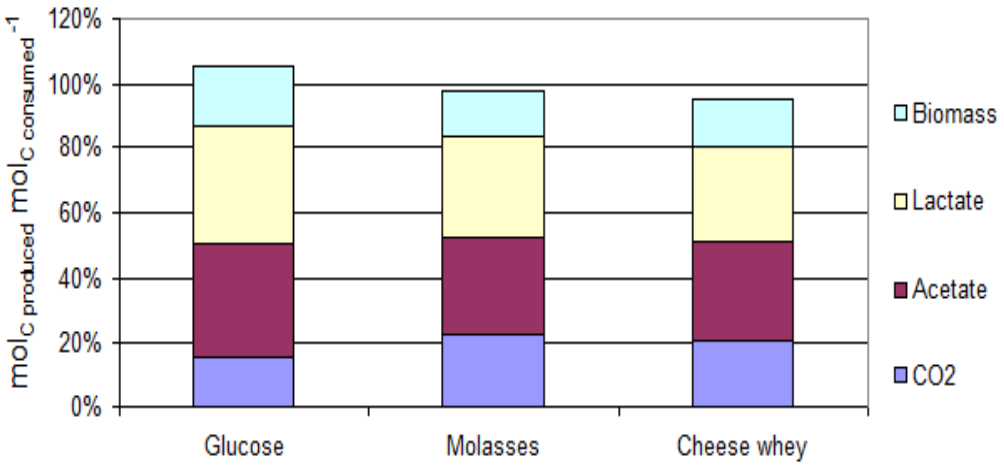

Figure 4.8 -Results of the application of the carbon balance to the results of *T. neapolitana* tests with suspended biomass.

The results for immobilized biomass are reported in Figure 4.9 only in terms of H_2 production rate to the 4 hours test. The tests was performed with immobilized biomass in 4 hours as described in Materials and Methods 3.4, every hour was analyzed the concentration of biogas produced. Indeed, $Y_{H2/S}$, $Y_{acetate/S}$ and $\eta_{E/S}$ were not considered reliable parameters for a 4-h immobilized-cell test, as the decrease in aqueous substrate concentration observed during the 4-h test is certainly affected by substrate diffusion in the biofilm. On the other hand, the attached-cell test could not be significantly lengthened to avoid excessive increase of suspended cells. The profiles of H_2 and CO_2 volumetric production versus time are reported in Figures 4.10 and 4.11.

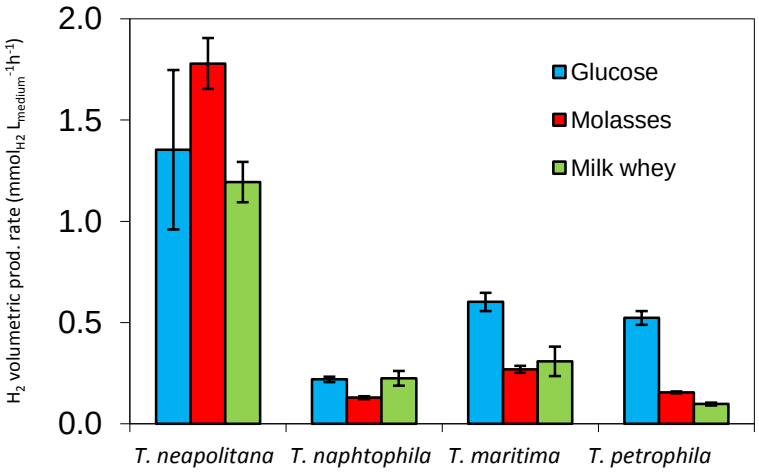

Figure 4.9 - H_2 production performances obtained with four Thermotoga strains immobilized tests.

In terms of H_2 production rate, *T. neapolitana* was markedly superior to the other three strains with all three substrates tested, as we can see in Figure 4.9. In the test with *T. neapolitana*, molasses showed a better yield about 1.8 mmol H_2/L_{medium}, glucose and milk whey has a very similar yield about 1.4 mmol H_2/L_{medium}.

Figures 4.10 and 4.11 confirm that *T. neapolitana* strain is better for H_2 production with 3 substrates. A comparison between suspended and immobilized cells of the four strains in terms of H_2 production cannot be performed on the basis of the results while the suspended cell H_2 rates were calculated during the exponential growth phase of cultures with low initial low cell concentrations (10–20 $mg_{protein}L^{-1}_{medium}$), the immobilized cell rates were obtained from the initial 4–6 hours of tests with higher cell concentrations (50–110 $mg_{protein}$ $L^{-1}_{packed\ reactor}$). The immobilized cell tests were, in fact, aimed at evaluating the feasibility of H_2

production by biofilms of the four Thermotoga strains, and at choosing the best-performing strain.

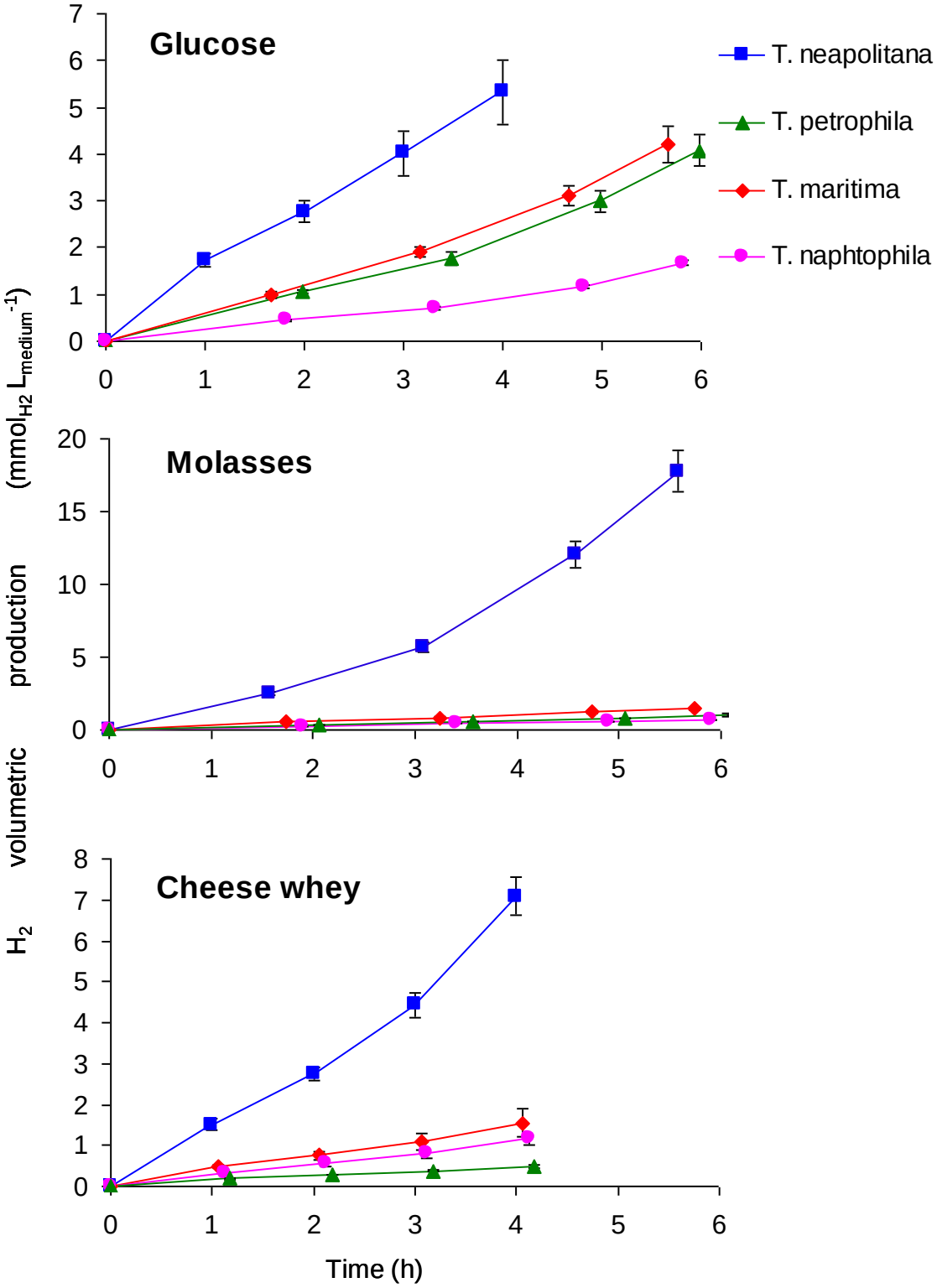

Figure 4.10 -Time profiles of volumetric H_2 production obtained with the immobilized cells of the 4 *Thermotoga* strains. Average data in each triplicate series ± standard deviations.

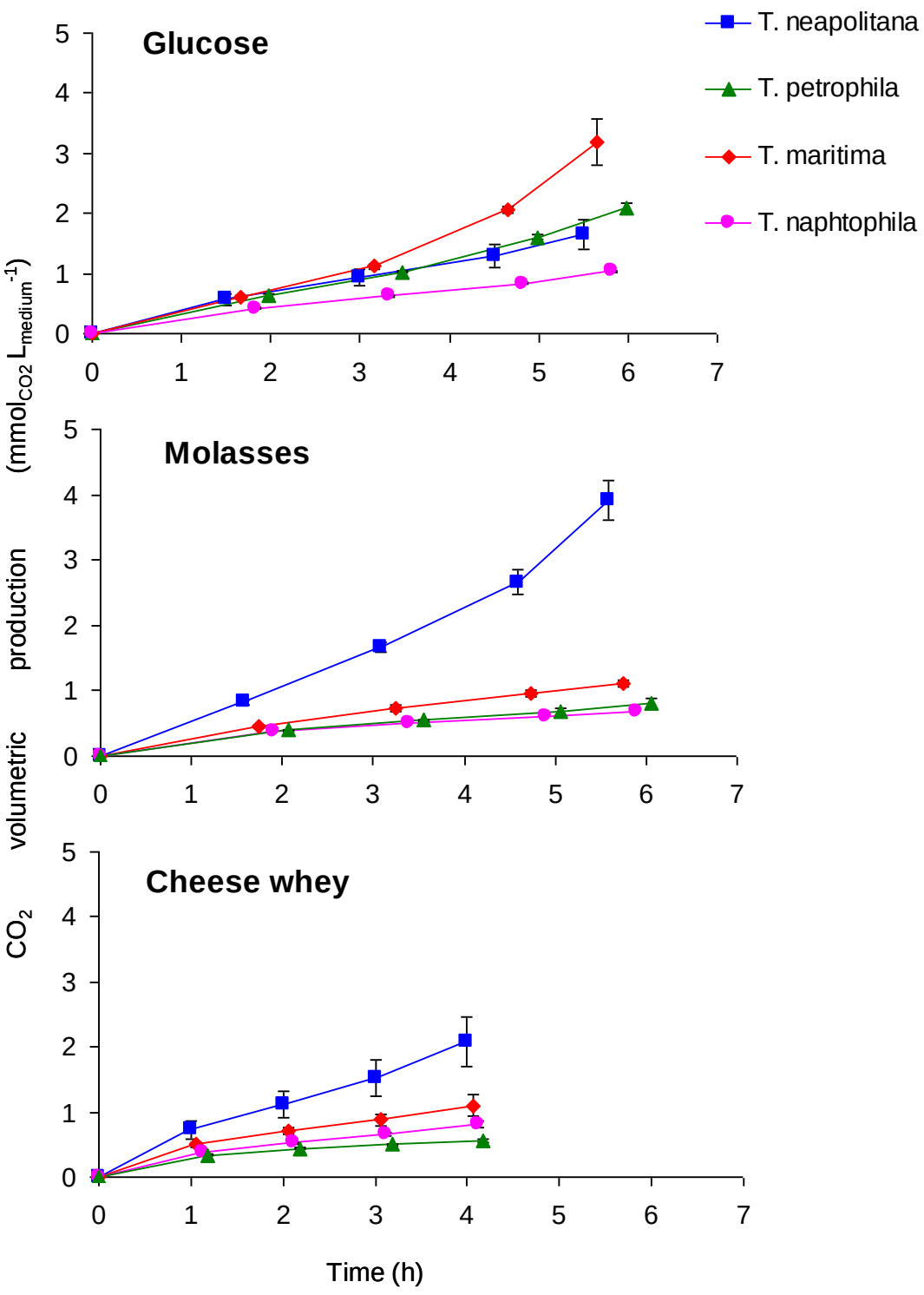

Figure 4.11- Time profiles of volumetric CO_2 production obtained with the immobilized cells of the 4 *Thermotoga* strains. Average data in each triplicate series ± standard deviations.

4.4 MINIMIZING THE MEDIUM COST

Minimal media for a more economic hydrogen production by *T. neapolitana* from molasses and whey were investigated. *T. neapolitana* was selected as the most efficient microorganism on the basis of the results obtained in previous tests. modATCC 1977 medium components was supplemented with whey and molasses as previously described (Materials and methods 3.3.3). Main modATCC 1977 components (microelements, vitamins, TSB, YE, and cysteine) were omitted step by step in order to maintain an efficient hydrogen production from more simplified media.

4.4.1 Molasses

Media chemical composition for tests carried out on modATCC 1977 media supplemented with molasses is shown in material and methods (Table 3.5) for tests with cysteine, in the (Table 3.6) for the test with metabisulfite (MBS). Hydrogen, carbon dioxide, biomass and acetate production by *T. neapolitana* from the medium tests are summarized in Table 4.4. The test 1 in the Table 4.4 is complete medium.

Data of the some tests carried out with MBS as a reducing agent are summarized in Table 4.5 we report the behavior of volumetric H_2 production against time for the best result in the Table 4.4

Table 4.4 - Results of the assays aimed at the optimization of the medium composition with molasses (cysteine).

Test	mmol H_2/L_{medium}	H_2/S yield (mol/mol)	Acetic acid/ S yield (mol/mol)	X/S yield (mg/g)	CO_2/S yield (mol/mol)	Substrate conversion (%)
1	120.1	2.64	1.5	75	2.1	86
2	76.64	2.51	0.744	75	1.14	52
3	80.92	3.24	1.027	82	1.54	43
4	83.75	3.02	1.15	107	1.6	44
5	83.23	3.14	1.076	104	1.59	45
6	32.13	2.32	0.15	70	3.6	28
7	104.7	3.37	1.70	35	1.8	55
8	72.60	3.815	2.175	93	1.99	41
9	25	1.3	0.021	40	0.38	24
10	78.00	2.71	1.54	55	1.56	60

Table 4.5 - Results of the assays aimed at the optimization of the medium composition with molasses (MBS).

Test	mmol H_2/L_{medium}	H_2/S yield (mol/mol)	Acetic acid/ S yield (mol/mol)	X/S yield (mg/g)	CO_2/S yield (mol/mol)	Substrate conversion (%)
1	20	1	0.9	23	0.6	47
2	42	2	1.2	24	1.1	47
3	55	2.3	0.31	40	0.4	66
4	49	2	0.31	18	0.75	67

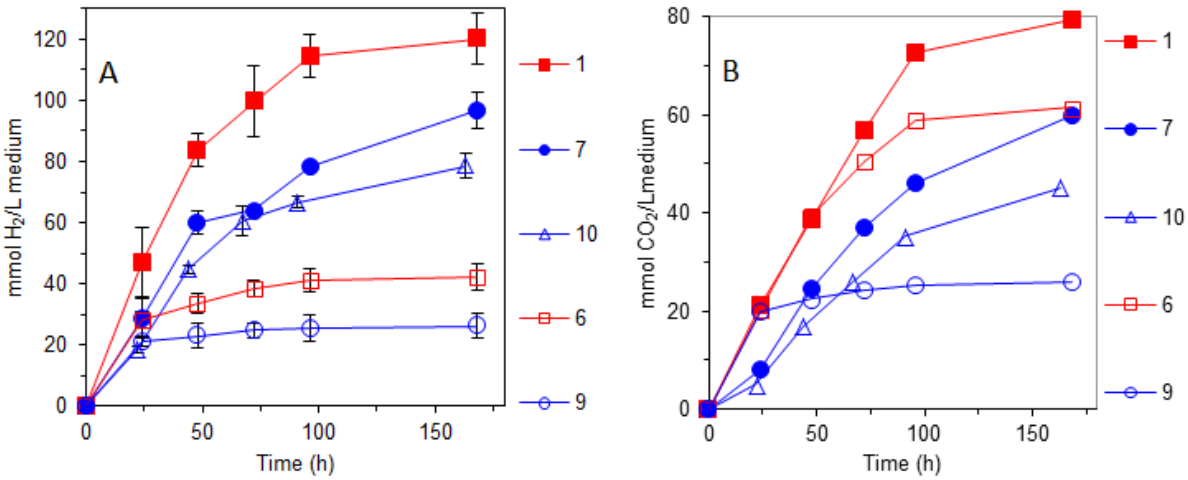

Figure 4.12 - The best results in test the Table 4.4 with cysteine as the reducing agent. (A) H_2 volumetric production, (B) CO_2 volumetric production.

Results of the test using cysteine as reducing agent showed that the use of a reducer is fundamental to the H_2 production; removing cysteine from the complete medium there was a reduction of 73% of the production of H_2, therefore cysteine is fundamental for the production H_2. Macroelements, that included NH_4Cl as inorganic nitrogen source, K_2HPO_4 as phosphate source and NaCl as osmotic pressure balance, were considered essential for microbial growth. Interestingly, no one of the main components of the medium (vitamins, microelements, TSB and YE) was found to be essential for hydrogen production by *T. neapolitana*.

Figure 4.13 illustrates the tests performed with the medium 7 of the Table 4.4 just by changing the reducing agent, cysteine by MBS. The medium 7 is the medium more promising.

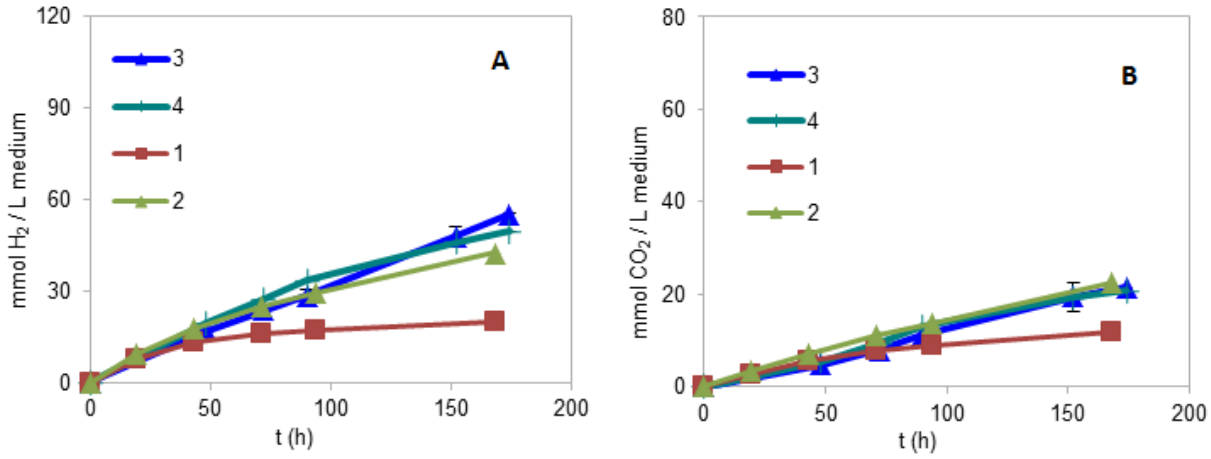

Figure 4.13 - The results in test the Table 4.5 with MBS as the reducing agent. (A) H_2 volumetric production, (B) CO_2 volumetric production.

The tests performed with MBS aimed at reducing costs of culture medium: the cysteine is one of the most expensive components, 100g cysteine costs about 62 euros, and 100g MBS cost 3.92 Euros. However as we can see in Figure 4.13 tests with MBS as reducing agent were not satisfactory, since the maximum production achieved with MBS was 55 mmolH_2/L_{medium} as those obtained with Cysteine was 96 mmol H_2/L_{medium}.

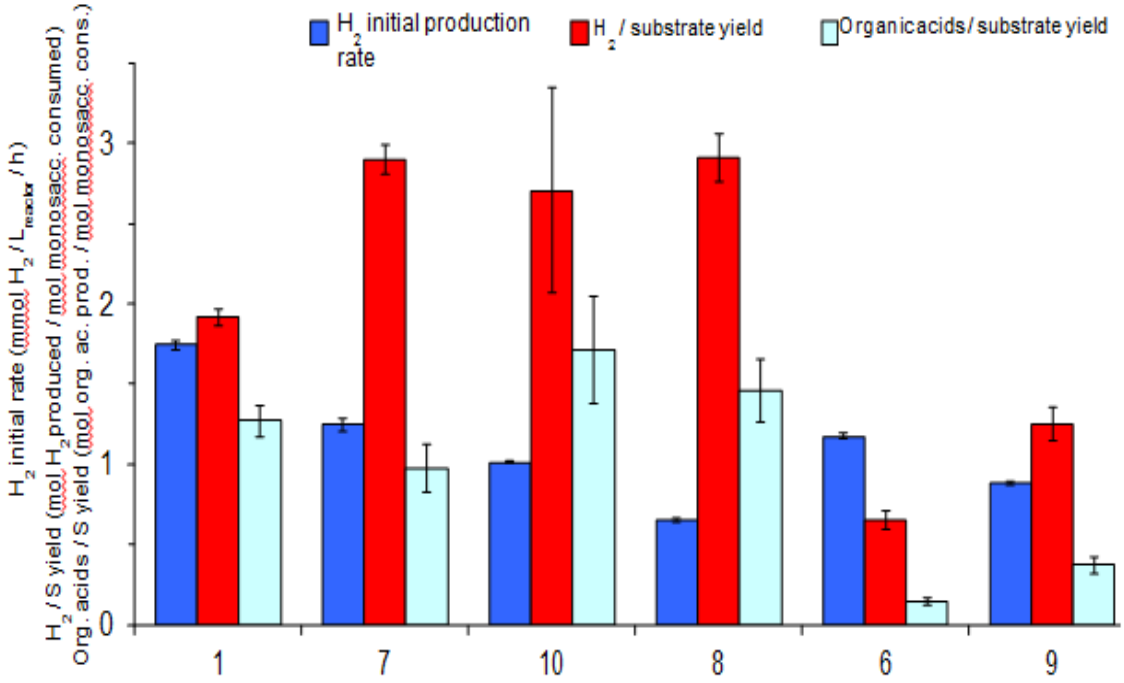

Figure 4.14 - Summary of tests conducted to reduce the culture medium molasses.

Figure 14.4 we have a small summary, of the tests realized for the minimization culture medium with molasses, with the most interesting data obtained during the tests. The tests perfomed with MBS are not show in Figure 4.14, as they were not satisfactory. For the media performance comparison, three parameters were used: rate initial production of H_2, H_2/S and acids/S.

After comparing all the results of the tests for the minimization of the culture medium for molasses, came to the conclusion that the best choice is medium 7 as it doesn't significantly alter the H_2 production but it reduces the cost of the medium around 90%. The medium composition is shown in Table 4.6. We can see in Table 4.4 dates of complete medium and minimal medium.

Table 4.6 – Minimal medium for Molasses.

Components	**g/L**
Hepes	23.8
NaCl	10
NH_4Cl	0.1
H_2PO_4	0.7
cysteine	1.0

The NaCl used in this study was coarse salt for cooking, which generated a greater reduction in the medium cost: 1kg NaCl analytical standard costs 75,60 Euros and NaCl to cook 0,50 Euro. This fact was further advantage of our study.

4.4.2 Milk whey

Media chemical composition for tests carried out on modATCC 1977 media supplemented with milk whey is shown in Table 3.7 for tests with cysteine, Table 3.8 for the test with MBS. Hydrogen, carbon dioxide, biomass and acetate production by *T. neapolitana* from the medium tests are summarized in Table 4.7.

Data of some tests with MBS as a reducing agent are reported in Table 4.8. In Figure 4.15 we have the behavior of volumetric H_2 production against time for the best results in the Table 4.7.

Table 4.7 - Results of the assays aimed at the optimization of the medium composition with milk whey (cysteine).

Test	mmol H_2/L_{medium}	H_2/S yield (mol/mol)	Acetic acid/ S yield (mol/mol)	X/S yield (mg/g)	CO_2/S yield (mol/mol)	Substrate conversion (%)
1	90.40	2.4	1.0	77	1.8	58
2	61	1.9	1.0	54	1.3	39
3	60	1.8	0.9	52	1.4	42
4	86	2.2	0.85	25	1.72	55
5	85	2.5	1.0	24	1.7	53
6	82.87	2.1	0.93	57	1.37	60
7	73.6	1.96	0.779	35	1.08	66
8	2.58	0.02	0.01	10	0.01	12
9	84	1.99	0.86	34	0.06	72
10	10.63	0.25	1.057	0.8	0.15	70
11	66.6	1.5	1.5	41	0.8	69
12	68.73	2.2	1.2	38	1.5	73
13	50.90	1.1	0.16	34	0.5	74
14	62	2.1	0.3	31	0.23	79
15	60	2.0	0.25	30	0.22	76

Table 4.8 - Results of the assays aimed at the optimization of the medium composition with molasses (MBS).

Test	mmol H_2/L_{medium}	H_2/S yield (mol/mol)	Acetic acid/ S yield (mol/mol)	X/S yield (mg/g)	CO_2/S yield (mol/mol)	Substrate conversion (%)
1	0.12	0.38	0.28	86	0.24	82
2	0.28	0.82	0.49	24	0.5	69
3	0.109	0.00	0.02	67	0.94	74
4	0.78	0.02	0.02	31	0.89	70

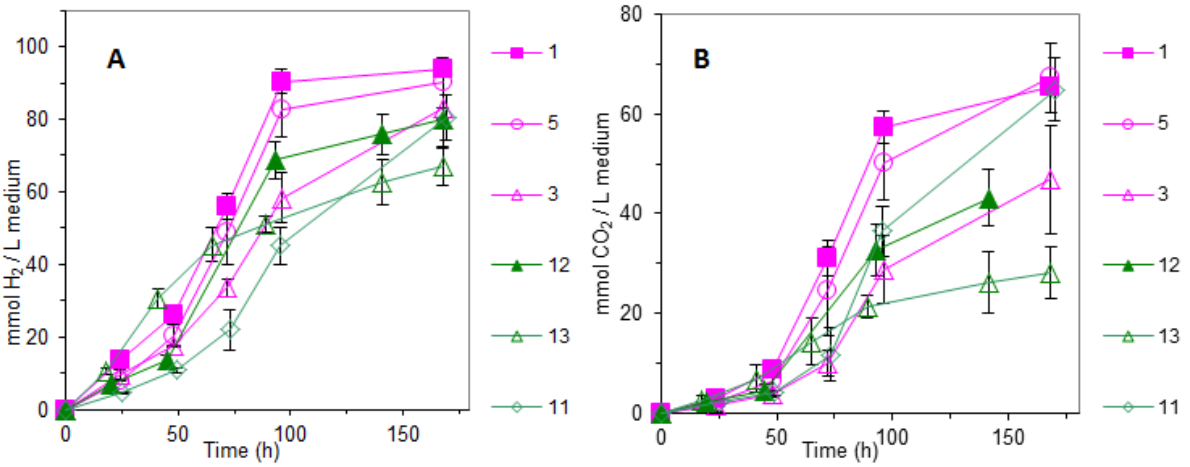

Figure 4.15 - The best results in test the Table 4.7 with cysteine as the reducing agent. (A) H_2 volumetric production, (B) CO_2 volumetric production.

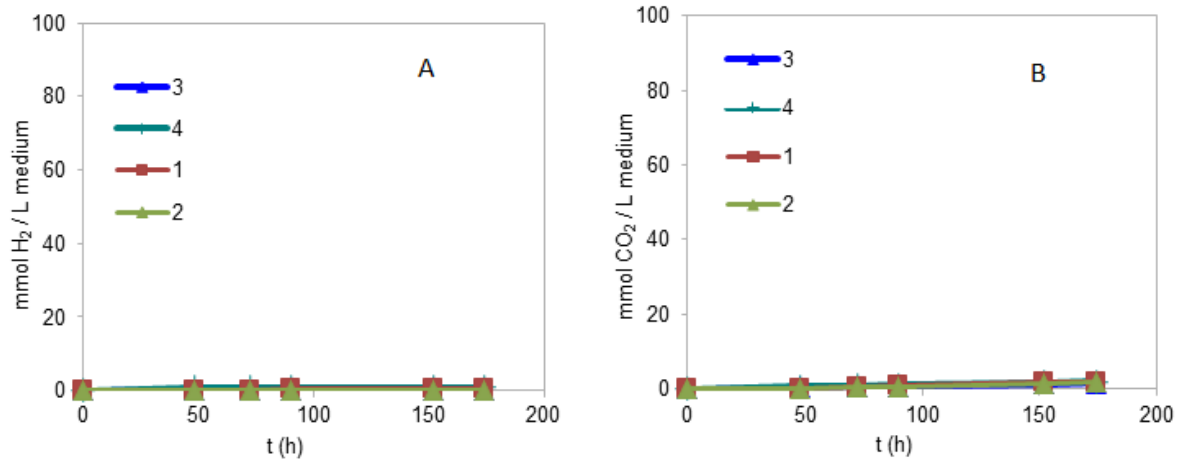

Figure 4.16 - The results in test the Table 4.8 with MBS as the reducing agent. (A) H_2 volumetric production, (B) CO_2 volumetric production.

The use of MBS as the reducing agent to the medium supplemented with milk whey was not satisfactory, the results were worse than those obtained with molasses.

Table 4.9 – Minimal medium for Milk whey.

Components	**g/L**
Hepes	23.8
NaCl	10
NH_4Cl	0.1
H_2PO_4	0.7
TSB	2.0
cysteine	1.0

Figure 4.17 we can see that the medium with the best performance was not obtained in the same tests with molasses, the best medium was medium12, as the similar the molasses

minimal medium but with an addition of TSB. The minimal medium for milk whey is on the Table 4.9. The minimal medium gave a 60% reduction in the cost value of the medium.

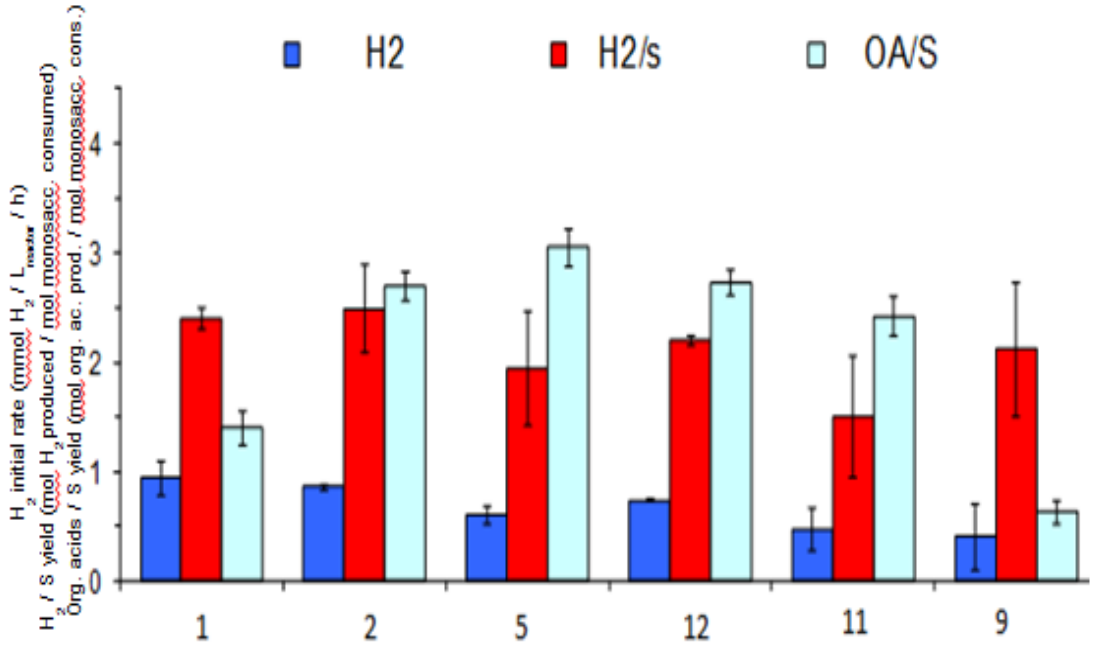

Figure 4.17 - Summary of tests conducted to reduce the culture medium milk whey.

4.5 POROUS CARRIER FOR BIOFILM FORMATION

Preliminary studies were carried out to compare porous carrier that permitted an efficient biofilm formation and an efficient hydrogen production by *T. neapolitana*. The four porous carrier tested are characterized by small dimensions (0.3-1 cm) and a high intrinsic empty volume. In addition, their large pore size is potentially efficient for biofilm formation.

Immobilized cells tests were carried out as described in Materials and Methods, Paragraph 3.4. Experiments lasted 3-4 hours, thus only 4-5 experimental points per test are available. R2 values obtained by linear regression of experimental set of data were higher than 0.95, thus, a linear interpolation could be considered to fit accurately experimental data in all the tests. Suspended cells at the end of the experiments were lower than 2.5% of the attached cells, thus, suspended cells contribution to hydrogen production could be considered negligible. Attached cells at the end point are at most 125% than initial ones. Hence, biomass could be considered constant throughout the tests.

T. neapolitana attached cells achieved the highest production rate (molar H_2 production per L of reactor) on *Biomax*® (Figure 4.18 and 4.19). Production rates on Biomax®, Glaxtone, Siporax cylinders and Siporax mini were respectively 1.354 mmol

H_2/L/h, 0.422 mmol H_2/L/h, 0.289 mmol H_2/L/h, and 0.012 mmol H_2/L/h. Hydrogen production rate by *T. neapolitana* cells attached on Biomax® was three-fold higher than those obtained from the growth on Glaxtone, Siporax cylinders, and Siporax mini as carrier.

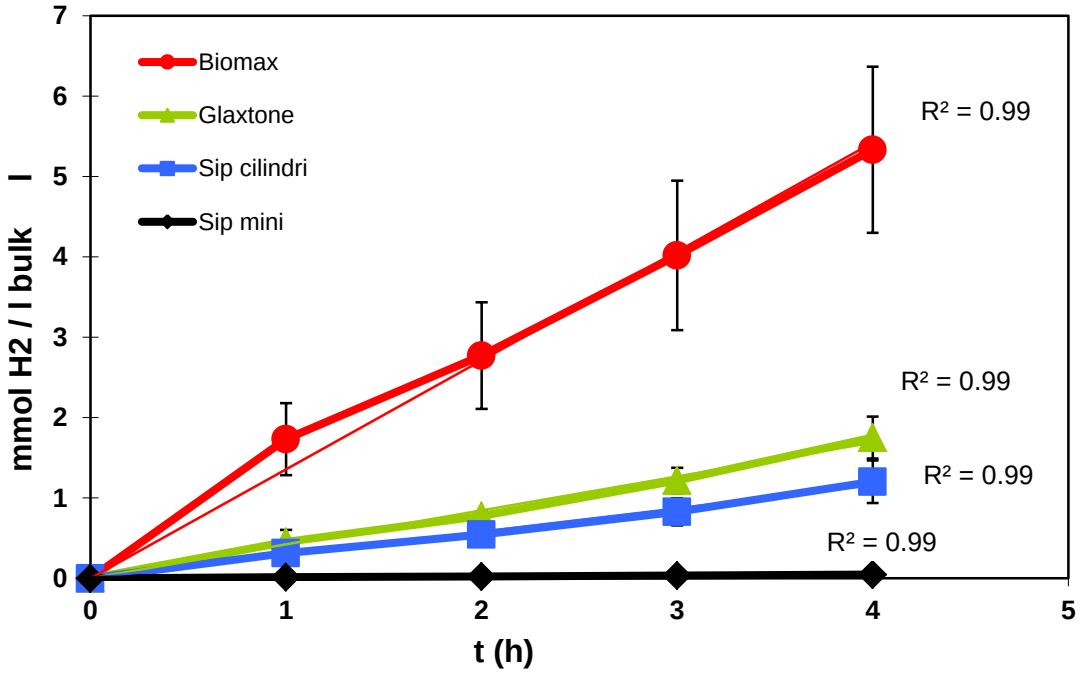

Figure 4.18 – Hydrogen production in batch cultures by *T. neapolitana* biomass attached on different porous carrier in the second phase of the experiment (4 hours). Red line, Biomax®; blue line, Siporax cylinders; green line,Glaxtone; black line, Siporax mini.

T. neapolitana attached cells on Biomax® achieved a final concentration of 10.5 g/L_{bulk}, that was two-fold higher compared to attached cells on Siporax cylinders (5.2 g/L_{bulk}) and Glaxtone (5.3 g/L_{bulk}), and higher compared to attached cells on Siporax mini (8.3 g/L_{bulk}). Thus, Biomax® permits higher concentrations of biomass respect to the other porous carrier.

Using the experimental volumetric production rate and the biomass concentrations data, the specific production rates (i.e. production rate normalized for attached biomass) were also calculated: 3.083 mmol H_2/g dry weight/d was obtained from *T. neapolitana* cultures grown on Biomax®, 1.948 mmol H_2/g dry weight/d on Glaxtone, 1.303 mmol H_2/g dry weight/d on Siporax Cylinders, and 0.035 mmol H_2/g dry weight/d on Siporax mini. Although differences among hydrogen specific production rates are lower, the value achieved by *T. neapolitana* cells grown on Biomax® (3.083 mmol H_2/g dry weight/d) was higher than those obtained from *T. neapolitana* cells grown on Glaxtone (1.948 mmol H_2/g dry weight/d),

Siporax cylinders (1.303 mmol H_2/g dry weight/d), and Siporax mini (0.035 mmol H_2/g dry weight/d).

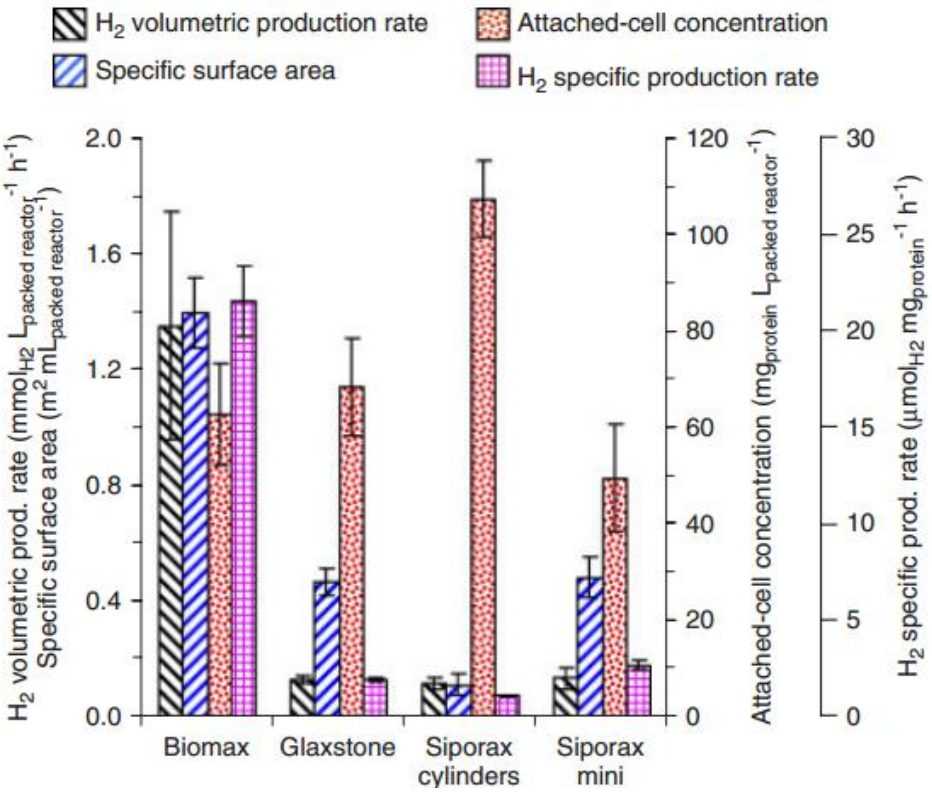

Fig. 4.19 – Porous carrier comparison.

In conclusion, *T. neapolitana* cells attached on Biomax® achieved the best results in terms of total attached biomass, hydrogen volumetric production rate, and hydrogen specific production rate. A further consideration takes into account the fact that Biomax® is the only ceramic carrier, while Glaxtone, Siporax cylinders and Siporax mini are chemical syntherized glass carrier. The combination of chemical composition and physical parameters (e.g. porosity, pore size) in Biomax® may be the reason for the success of this support. Biomax® was selected for further experiments investigating hydrogen productions by *Thermotoga* species grown as biofilm.

4.6 OPTIMAL pH FOR H_2 PRODUCTION BY *T. NEAPOLITANA* UNDER BIOFILM CONDITIONS.

The following immobilized biomass growth tests were performed with *T. neapolitana* cultivated on medium ATCC 1977 and fed with glucose (7.5 g/L) as the carbon source. The tests were performed as described in section 3.3 of Materials and Methods. In the second stage of the experiment, the fresh medium pH corrected to different values, namely 5, 5.88,

6.75, 7.63, 8.5 and 9.38 was added to the samples containing carriers with immobilized biomass and maintained in an atmosphere of nitrogen.

Figure 4.20 describes the investigation carried out on H_2 production by *T. neapolitana* to the optimal growth temperature, in a soil at different pH values. When the value of initial pH increases from 5 to 7, there is an increase in the production rate of hydrogen, from 0.065 to 0.077 $mmolH_2L^{-1}h^{-1}$; the increase in the initial pH in the range between 7.63 and 8.5 result in the achievement the maximum value of speed of production of H_2 initial (0.087 $mmolH_2L^{-1}h^{-1}$) that decreases in the soil with initial pH equal to 9.38.

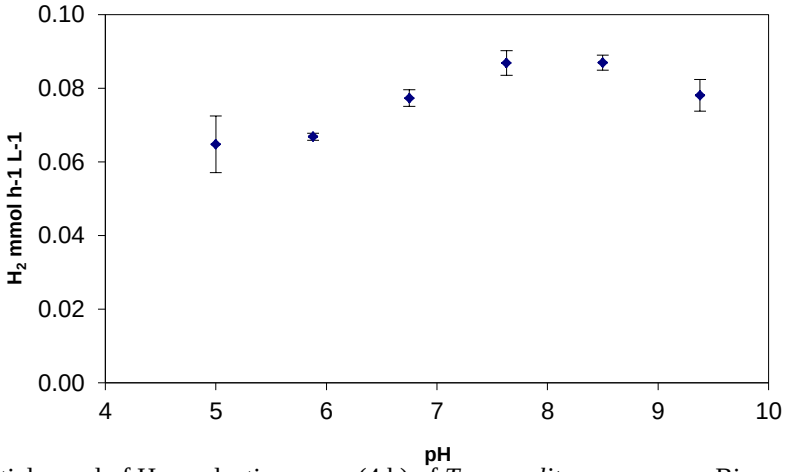

Figure 4.20 - Initial speed of H_2 production assay (4 h) of *T. neapolitana* grown on Biomax® in medium with glucose.

In Figure 4.21 the values of production rate of H_2 are report together with associated substrate production yields; similarly to the values of rates, even in this case the maximum yield is obtained the interval of initial pH of 7.6 - 8.5. The results show a significant effect of different values of initial pH on the productivity of *T. neapolitana* in terms of hydrogen production and conversion of the substrate provided for the growth; this implies the fact that the control of this parameter is critical to the process of production of H_2 by this microorganism.

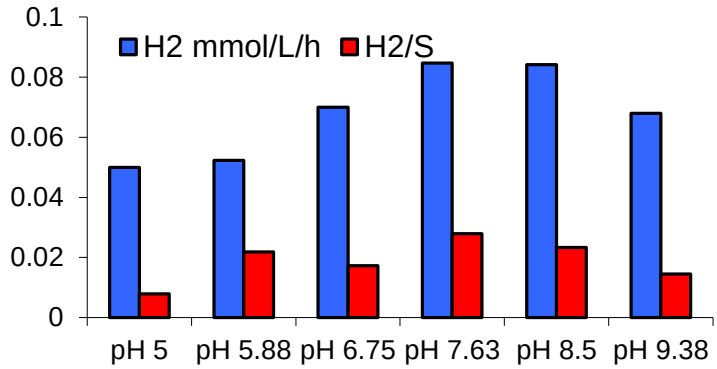

Figure 4.21 - Initial speed of production of H_2 (test 4h) of *T. neapolitana* grown on Biomax® in medium with glucose.

4.7 INHIBITION OF OXYGEN

T. neapolitana is an aerobic bacterium, however during the test it is possible that small quantities of O_2 penetrate the reactor, which may be through the pH control system, by reactor leakages, through the culture medium during continuous tests. Some kinetic tests were carried to evaluate the possible damage to H_2 production due to the presence of O_2 in the reactor. The test procedure is described in Materials and Methods 3.5.1, tests were performed only for milk whey that is the substrate chosen for the tests a 20 L reactor with suspended and immobilized biomass.

In Figure 4.22 we can see the test results in terms of rate specific production of H_2.

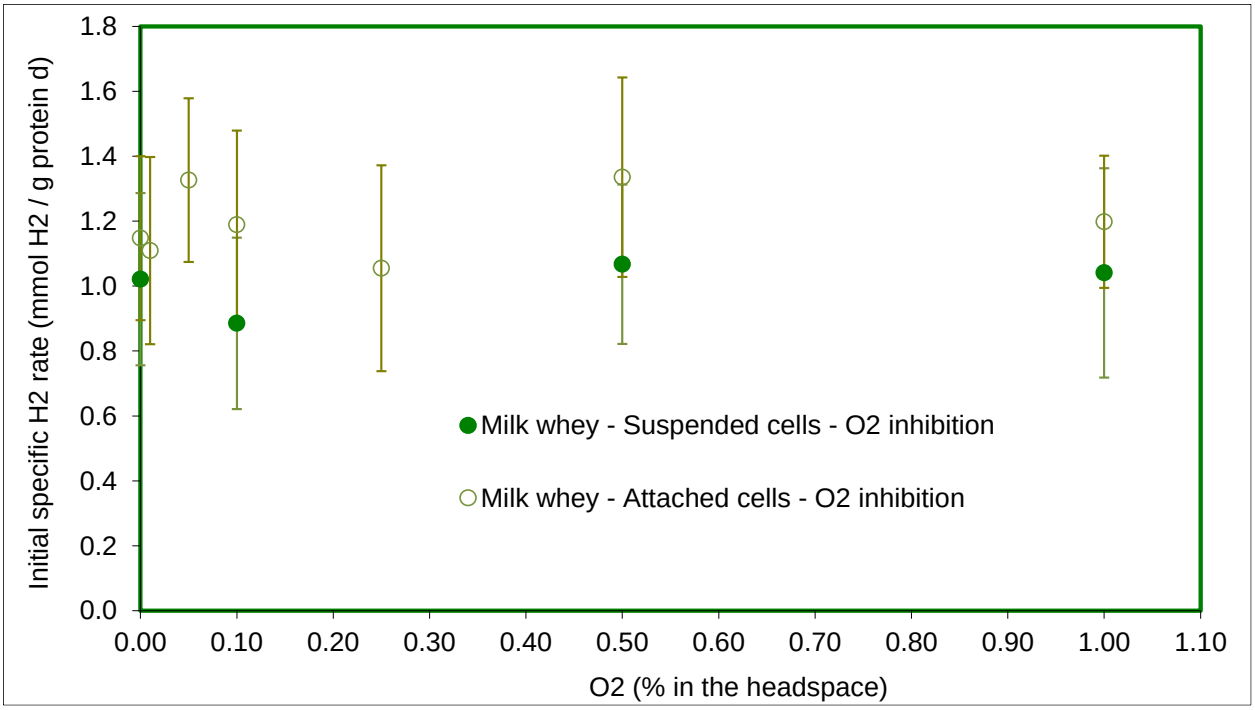

Figure 4.22 – Specific hydrogen production rate by *T. neapolitana* cells immobilized and Suspended on Biomax® at different initial oxygen concentrations in bottles head space.

The results in Figure 4.22 shown that H_2production is almost constant as O_2 concentration increases and that, therefore, both the suspended and the immobilized biomass are not inhibited by an oxygen content lower than 1% in the head space.

4.8 INHIBITION OF HYDROGEN

Product inhibition is an important parameter to be taken into consideration in fermentative hydrogen production. Even if hydrogen inhibition is supposed to be present in *T. neapolitana*, no study pointed out hydrogen concentrations that could inhibit further hydrogen production. Moreover, it is important to establish inhibiting hydrogen concentrations before of analyzing hydrogen production kinetics for the tested substrates.

The tests were performed with three substrates and under conditions of suspended and immobilized biomass as described in 3.5.2.

Figure 4.23 reports the results obtained with glucose as substrate in terms of initial specific H_2 rate versus H_2 concentration in the head space. The results show a significant difference between the specific rates measured with suspended and immobilized biomass, about 5 times larger in the case of immobilized biomass, with no evidence of H_2 inhibition for both the cases: the production rate remained constant with increasing hydrogen concentration in the head space.

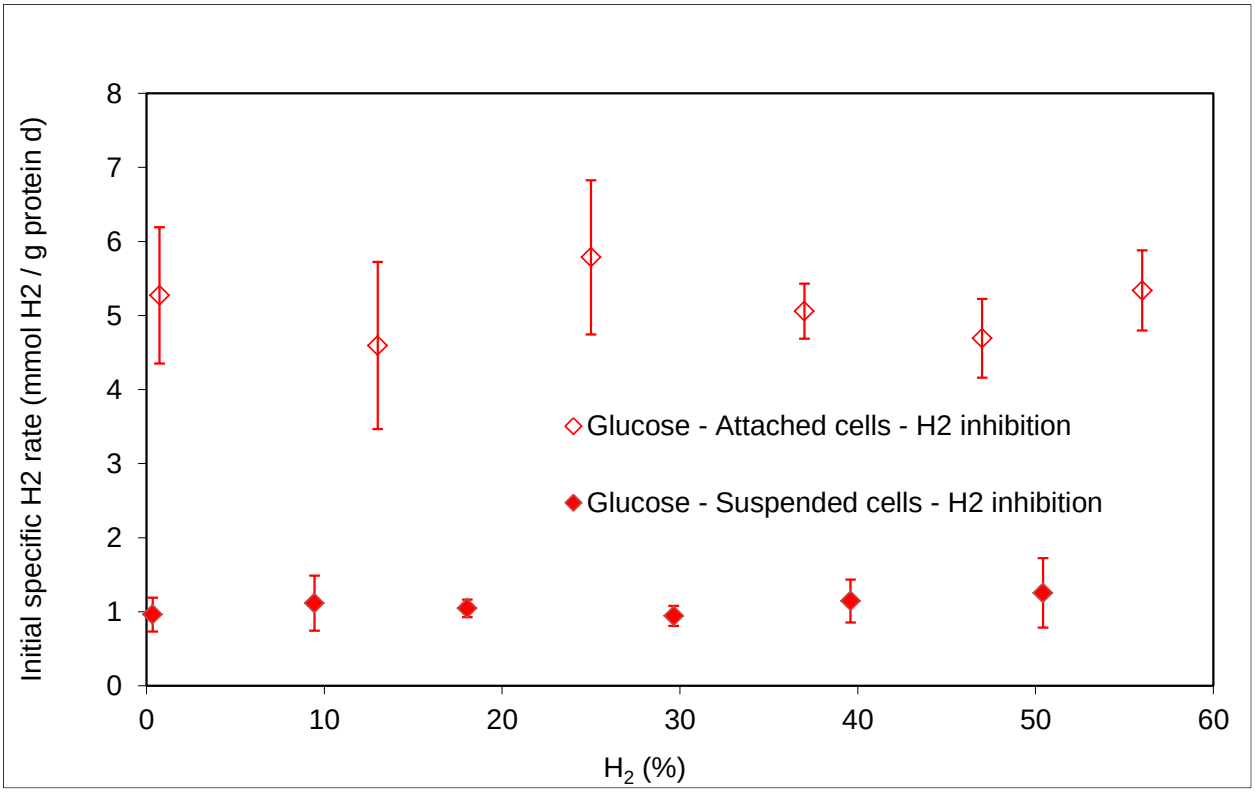

Figure 4.23 – Specific hydrogen production rate by *T. neapolitana* cells immolized and suspended on Biomax® at different initial hydrogen concentrations in bottles headspace for glucose with substrate.

In Figure 4.24 have the results for Molasses. Molasses also to have a difference in the specific rates for suspended and immobilized biomass, in this case the rates obtained with immobilized biomass is about two times higher. No H_2 inhibition effect was observed for suspended biomass up to 58% of H_2 in the head space and for immobilized up biomass to 38% of H_2.

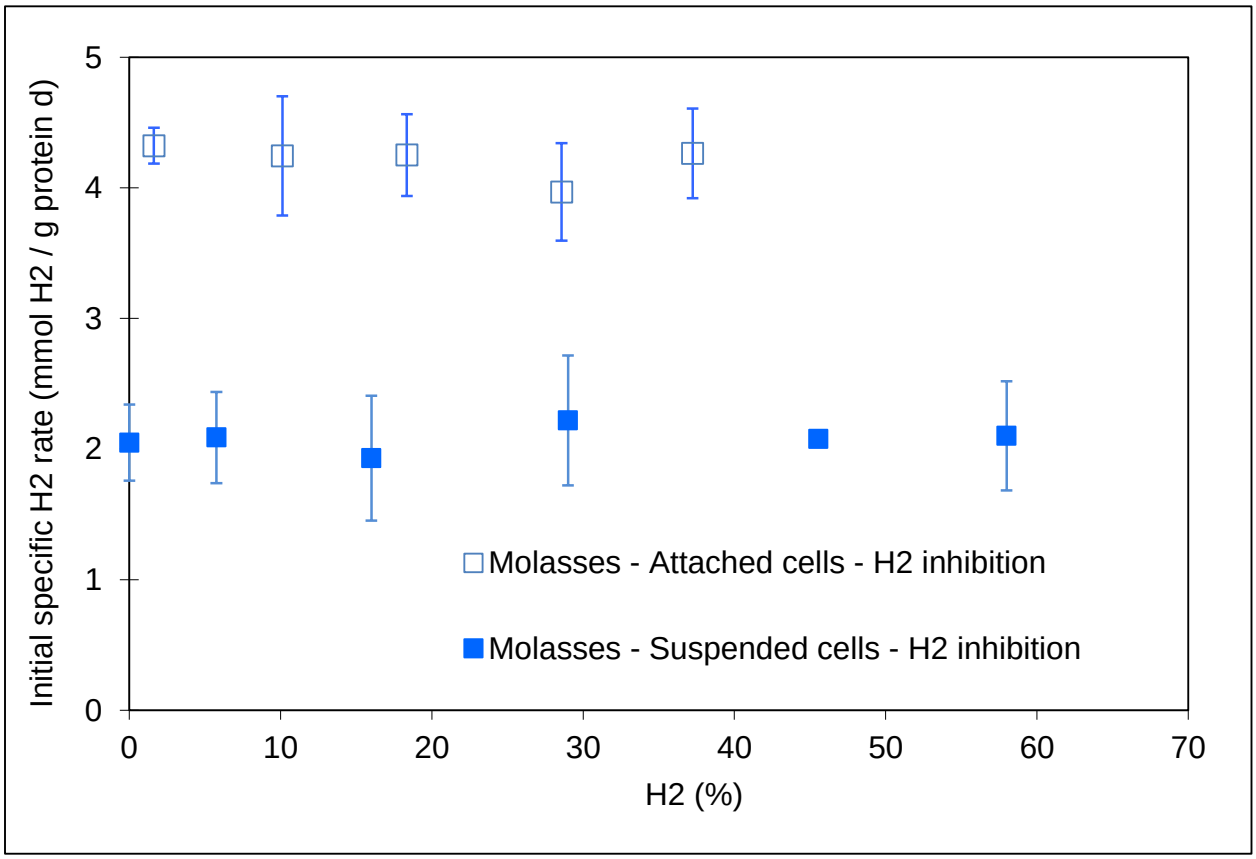

Figure 4.24 – Specific hydrogen production rate by *T. neapolitana* cells immobilized and suspended on Biomax® at different initial hydrogen concentrations in bottles head space for molasses with substrate.

Figure 4.25 in the case of milk whey no significant difference in the initial rates of specific H_2 for suspended and immobilized biomass was observed, the values were near those obtained with glucose and suspended biomass. The low initial specific H_2 rate obtained with immobilized biomass is probably due to substrate inhibition, which occurs in the case of whey; this issue will be addressed better in the next topic of the thesis. We can see that there was no H_2 production inhibition for milk whey.

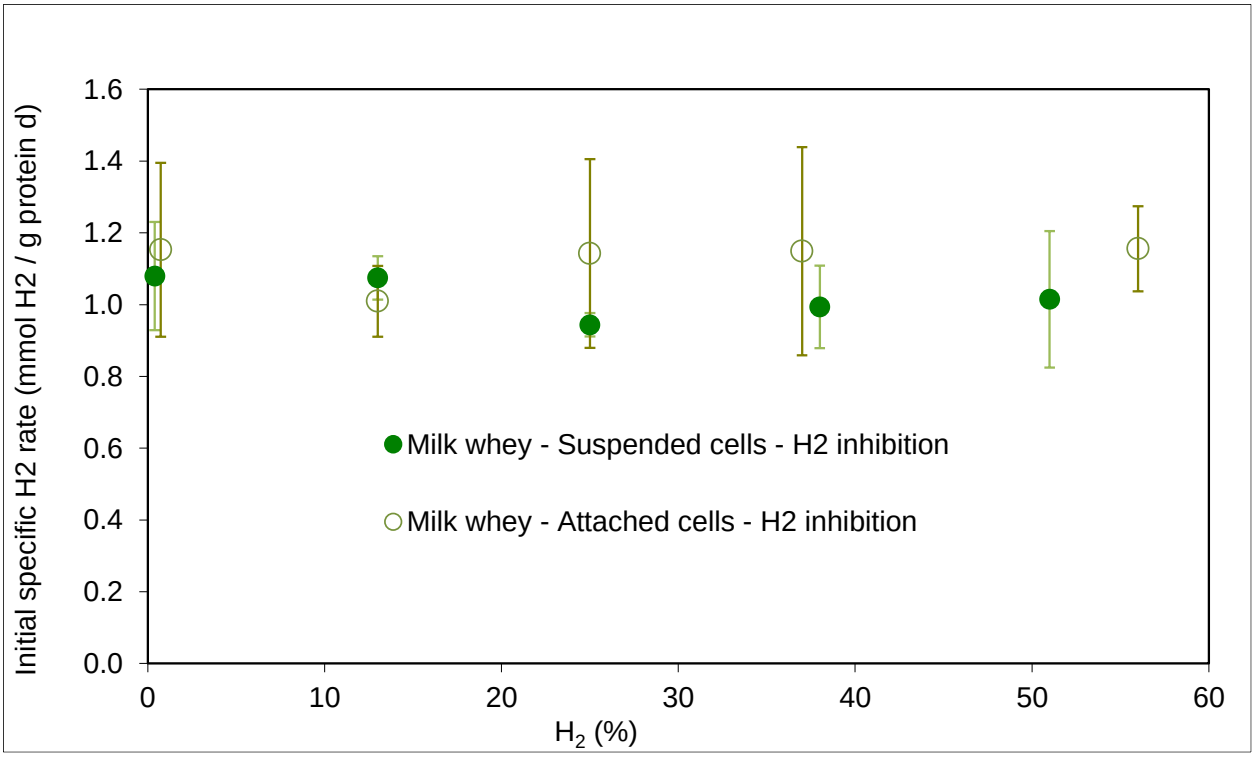

Figure 4.24 – Specific hydrogen production rate by *T. neapolitana* cells immobilized and suspended on Biomax® at different initial hydrogen concentrations in bottles head space for milk whey with substrate.

Table 4.10 is a summary of product inhibition tests.

Table 4.10 – Summary results of test Inhibition of H_2

Substrate	**Biomass Condition**	**Initial biomass mg/L**	**Initial. Spec. H_2 rate (mmol H_2/g prot h)**
Glucose 7.5 g/L	Immobilized	133	5.12
	Suspended	378	1.08
Molasses 20 g/L	Immobilized	128	4.21
	Suspended	341	2.08
Milk Whey 12.5 g/L	Immobilized	209	1.12
	Suspended	481	1.02

4.9 INHIBITION OF SUBSTRATE

In order to assess a suitable kinetic model and to estimate the values of substrate affinity constants (K_S), substrate inhibition constants (K_I) and maximum specific hydrogen production rates (q_{max}), the kinetics of hydrogen production by *T. neapolitana* from ATCC 1977 medium, Molasses minimal and Milk whey minimal were studied.

The kinetic model used is Haldane/Andrews with an inhibiting factor:

$$q_{H2} = \frac{q_{max,\ H2} \cdot S}{K_S + S + S^2 / K_I}$$

The Haldane/Andrews model is the most common and most used to describe bacterial growth under substrate inhibition. This model has proved appropriate for the data.

4.9.1 GLUCOSE

Different initial glucose concentrations in modATCC 1977 were used to investigate hydrogen specific production rate by *T. neapolitana* for both suspended and immobilized biomass; the conventional initial rate method was used as described in Material and Methods Table 3.11. The initial specific H2 production rates were evaluated; they are plotted versus the initial glucose concentrations in Figure 4.25. A zoom of Figure 4.25 is shown in Figure 4.26 (suspended cells) and 4.27 (immobilized cells). The kinetics parameters were estimated by means of a parameter optimization procedure based on the conventional non-linear least-squares method that minimized the summed square of residuals as the objective function. Best estimates of the kinetic model parameters are summarized in the Table 4.11 for both suspended and immobilized biomass.

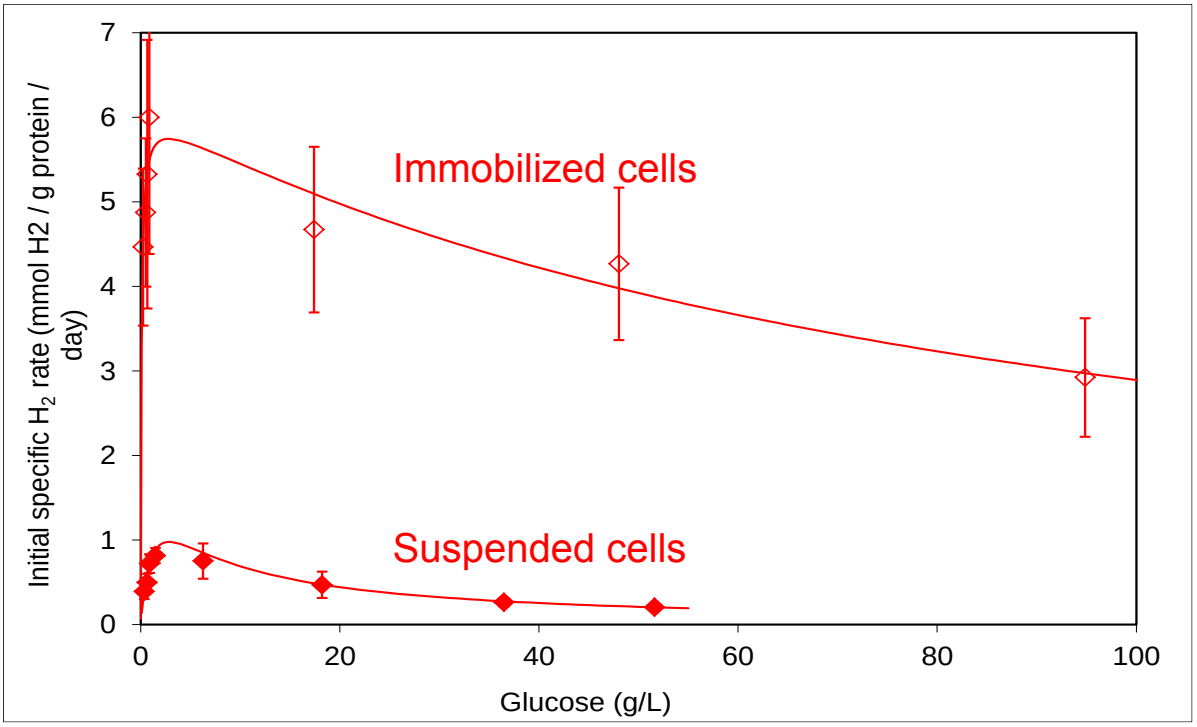

Figure 4.25 – Specific hydrogen production rate versus initial glucose concentration by *T. neapolitana* on ATCC 1977 media.

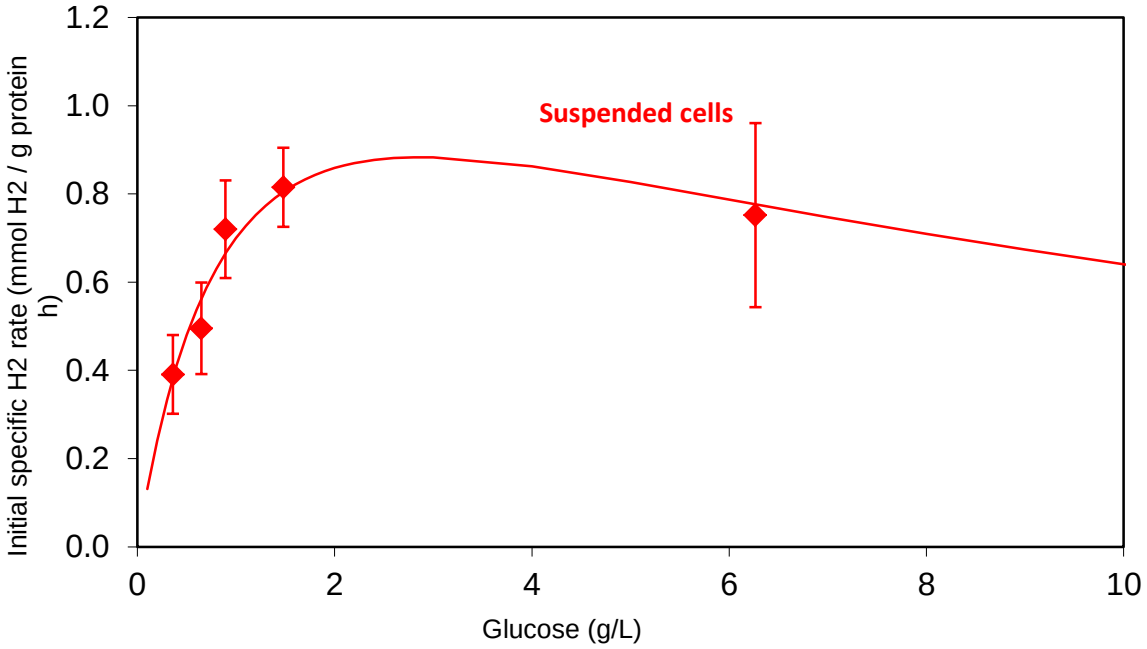

Figure 4.26 – Specific hydrogen production rate versus initial glucose concentration (0-6 g/l) by *T. neapolitana* on ATCC 1977 media. Zoom of Figure 4.25 for suspended cells.

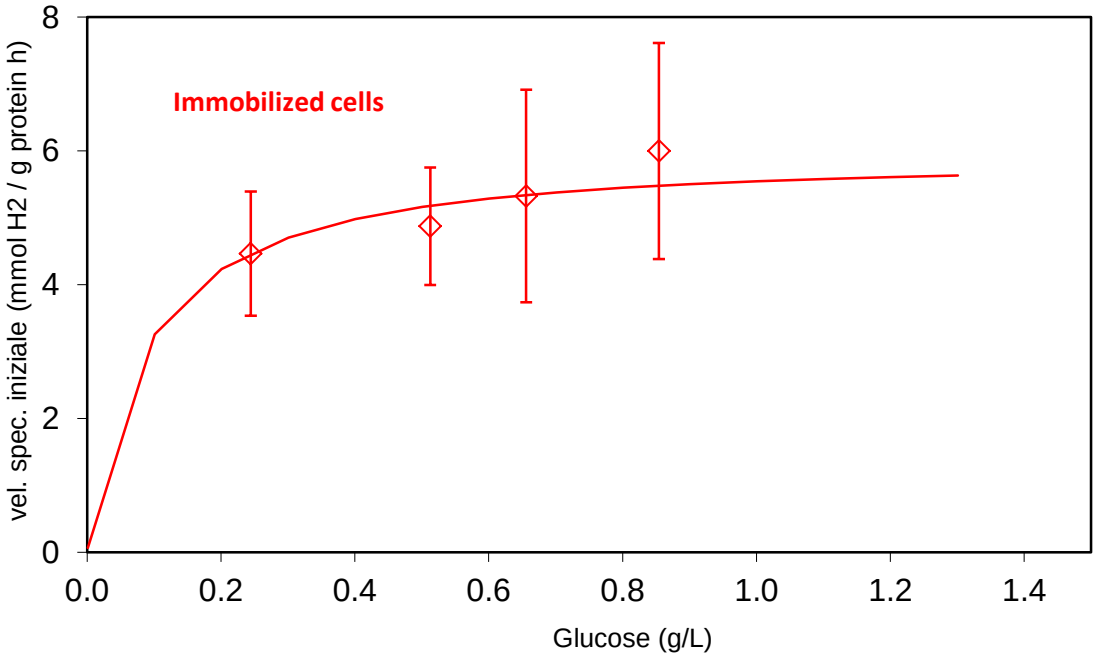

Figure 4.26 – Specific hydrogen production rate versus initial glucose concentration (0-1 g/l) by *T. neapolitana* on ATCC 1977 media. Zoom of Figure 4.25 for immobilized cells.

A substrate inhibition is present and that this inhibition is remarkably decreased in case of biofilm conditions.

Table 4.11 – Kinetic constants for glucose, molasses and milk whey.

	Cells conditions	**q_{maxH2}**	**K_s**	**K_i**	**S_{max}**	**S***
		($mmol\ H_2 / g_{protein} / h$)	**(g/L)**	**(g/L)**	**(g/L)**	**(g/L)**
Glucose	Immobilized	6.10	0.09	90.2	2.8	9.1
	Suspended	1.55	1.08	7.7	2.9	1.4
Molasses	Immobilized	7.99	0.64	12.0	2.8	1.7
	Suspended	2.21	0.22	31.6	2.7	3.4
Milk Whey	Immobilized	2.21	1.20	11.5	3.7	1.9
	Suspended	1.73	1.46	27.8	6.4	3.8

K_s values are minor than the K_i ones for both suspended and immobilized biomass; this is a good result, showing that *T.neapolitana* has a high affinity for the substrate, in particular for the glucose when cells are immobilized. The greater the difference between K_s and K_i, the greater is the affinity of the bacteria for the substrate. In the case of immobilized

biomass, the value of the K_s is about 1000 times lower than K_i, again emphasizing the importance of working with biofilm. qmax values reported are consistent with the values obtained in the other tests shown in this work.

4.9.2 MOLASSES

Different initial molasses concentration in Minimal medium for molasses employed to investigate hydrogen specific production rate by *T. neapolitana* were studied for suspended and immobilized biomass, the initial concentrations for molasses are described in material and methods. Table 3.11. Specific production rates evaluated are plotted versus initial glucose concentrations in Figure 4.28. A zoom of Figure 4.28 is shown in Figure 4.29 (suspended cells) and 4.30 (immobilized cells). Kinetics parameters calculated are summarized in the Table 4.11.

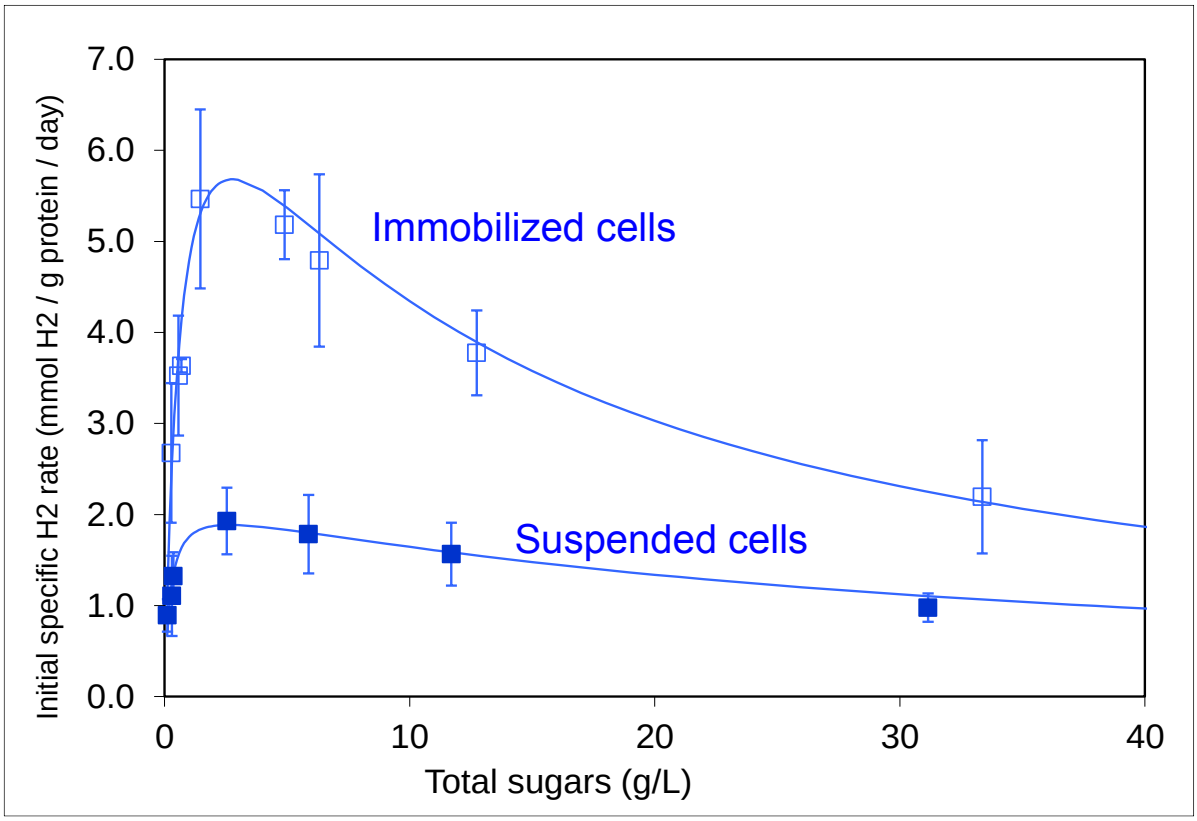

Figure 4.28 – Specific hydrogen production rate versus initial molasses concentration by *T. neapolitana* on Minimal medium for molasses**.**

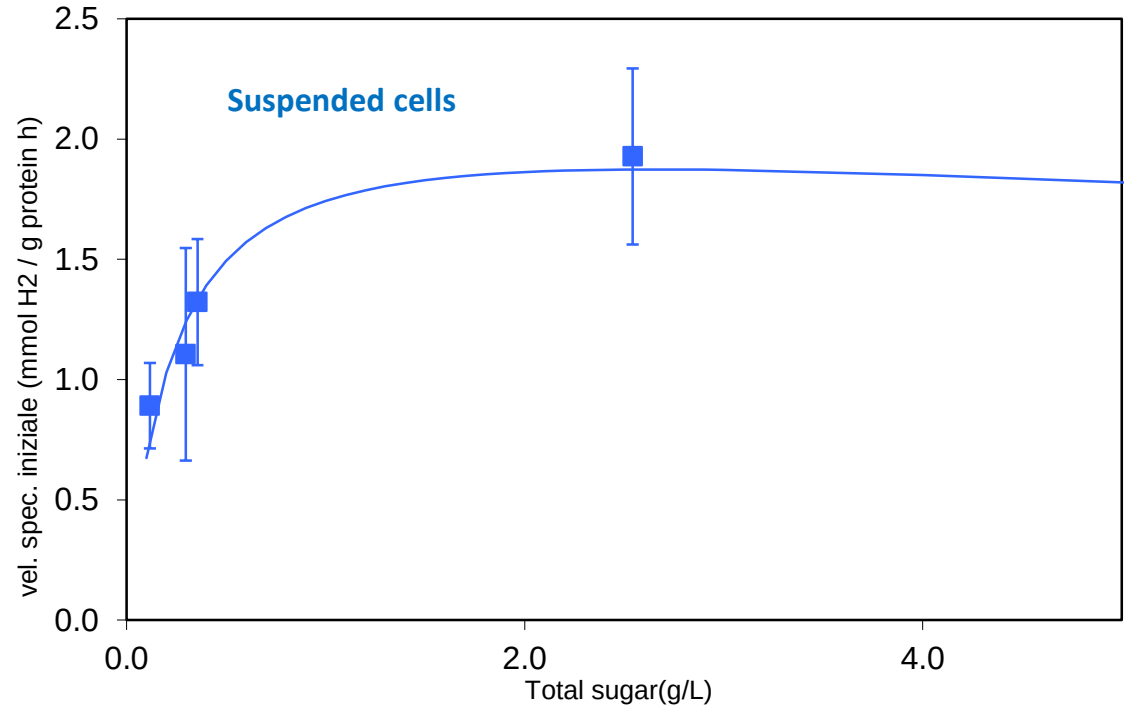

Figure 4.29 – Specific hydrogen production rate versus initial molasses concentration (0-2.5 g/l) by *T. neapolitana* on minimal medium for molasses. Zoom of Figure 4.28 for Suspended cells.

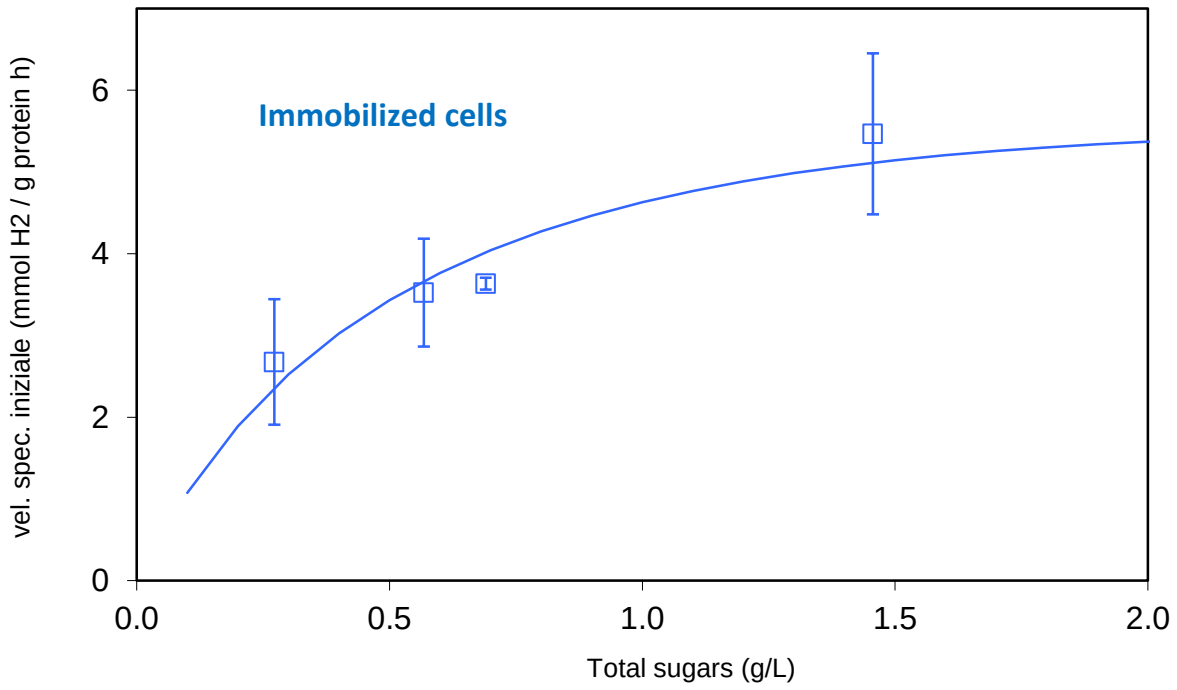

Figure 4.30 – Specific hydrogen production rate versus initial molasses concentration (0-1.5 g/l) by *T. neapolitana* on minimal medium for molasses. Zoom of Figure 4.28 for immobilized cells.

The data obtained with molasses are similar to those already shown anteriorly for glucose. Also for the case of molasses there is a lower substrate inhibition in the H_2 production if a biofilm is present. The kinetic constants obtained were calculated in the range of lower sugar concentrations, as seen in Figures 4.29 and 4.30.

Analyzing the Figure 4.28 it can be seen that the initial velocities obtained with immobilized biomass are still much higher than those obtained with suspended biomass. However, the value of K_i for suspended biomass is much higher than that obtained with immobilized biomass lower inhibition: in the case od suspended biomass the values of initial velocities remain almost constant after reaching the maximum K_S while increasing sugar concentration.

4.9.3 MILK WHEY

Finally the hydrogen specific production rates by *T. neapolitana*, under suspended and immobilized biomass conditions were studied also for milk whey using the minimal medium.

The specific production rates are plotted versus initial lactose concentrations in Figure 4.31. A zoom of Figure 4.31 is shown in Figure 4.32 (suspended cells) and 4.33 (immobilized cells). The corresponding estimated kinetics parameters are summarized in the Table 4.11.

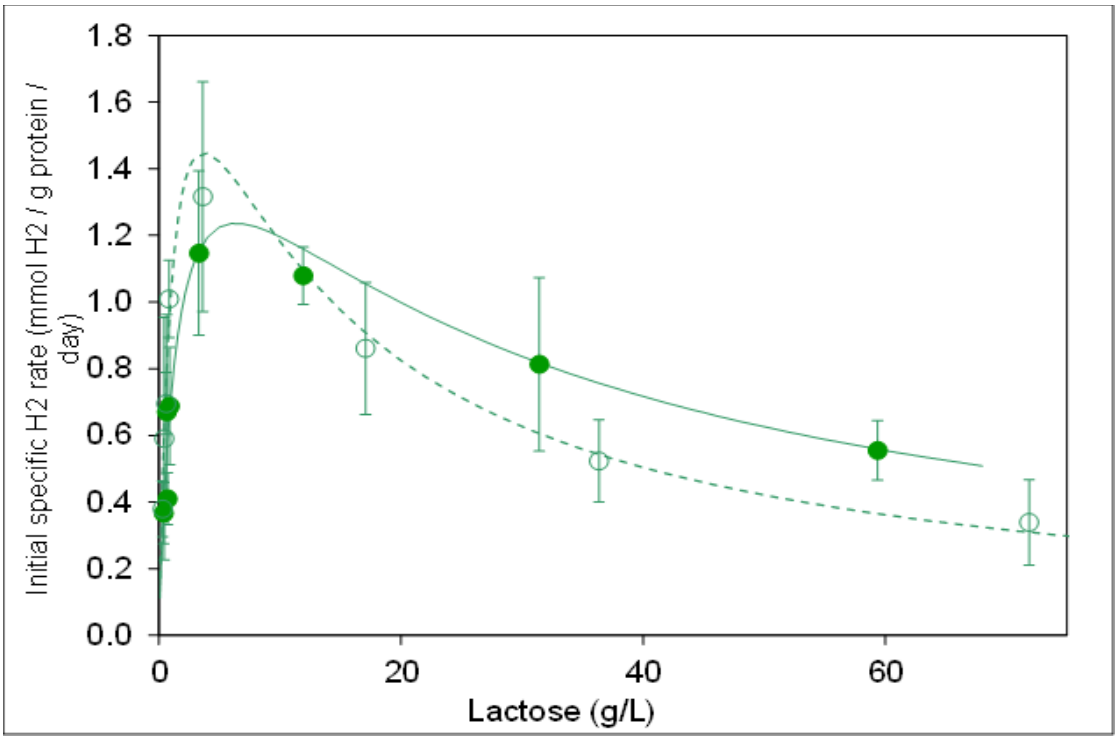

Figure 4.31 – Specific hydrogen production rate versus initial milk whey concentration by *T. neapolitana* on Minimal medium for milk whey**.**

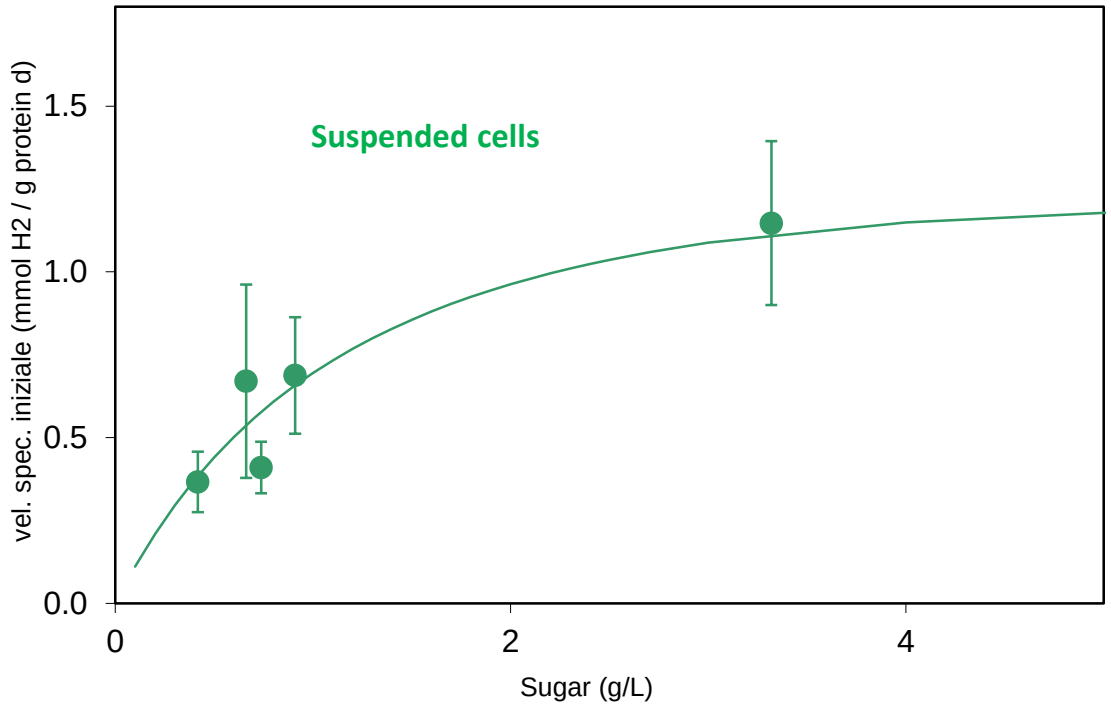

Figure 4.32 – Specific hydrogen production rate versus initial milk whey concentration (0-3.5 g/l) by *T. neapolitana* on minimal medium for milk whey. Zoom of Figure 4.28 for Suspended cells.

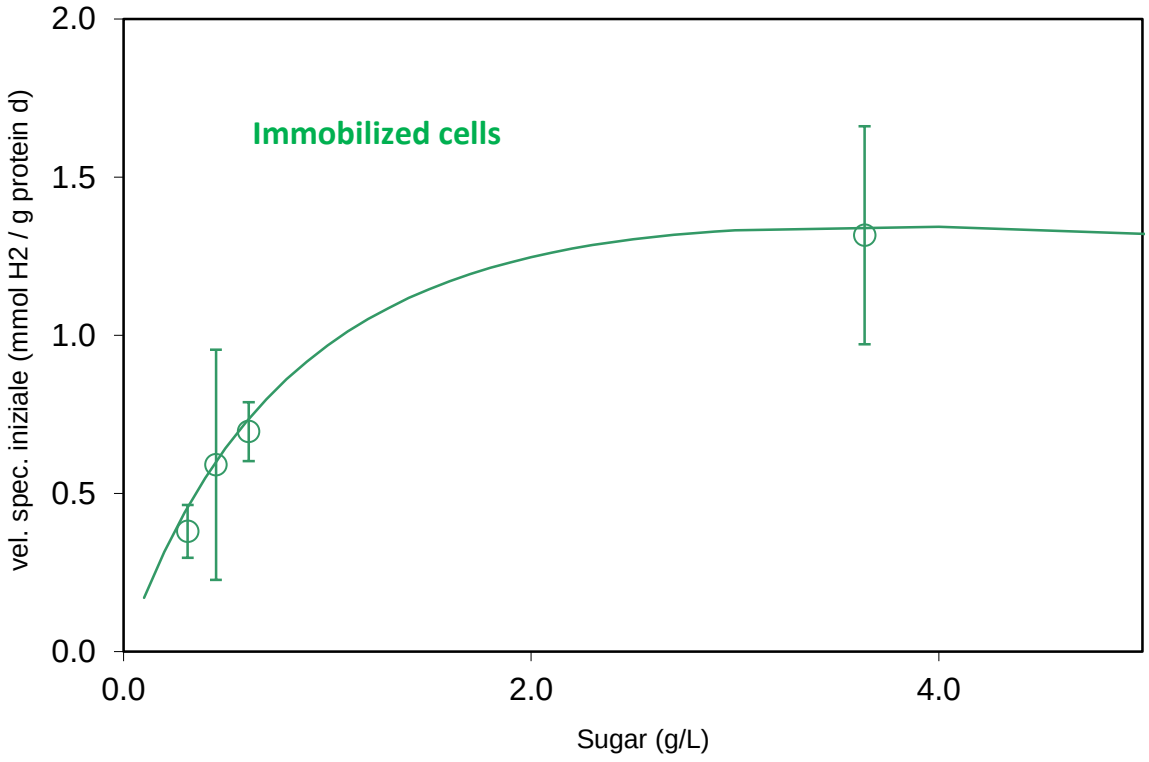

Figure 4.33 – Specific hydrogen production rate versus initial milk whey concentration (0-3.8 g/l) by *T. neapolitana* on minimal medium for milk whey. Zoom of Figure 4.28 for immobilized cells.

In the case of milk whey the behaviors of suspended and immobilized biomass were similar; the differences between the two curves are within the experimental errors and are not statistically significant. For the immobilized biomass there is a only slightly greater q_{max}, even if in the presence of a slightly higher substrate inhibition (lower K_I), a small difference that, however it is enough to yield higher H_2 production rate with immobilized biomass.

Through Table 4.11 and Figure 4.31 we can see a substrate concentration of about 10 g/L of milk whey is enough to exert a significant inhibition on *T. neapolitana*; this fact justifies the occurrence of low initial specific H_2rates obtained during testing of product inhibition. For concentrations lower than 10.1 g/L milk whey has better production when operating in conditions of immobilized biomass, this fact is very important for a later stage working in a 20 L reactor.

Using data obtained from the above evidence with suspended and immobilized biomass was developed Lumped model:

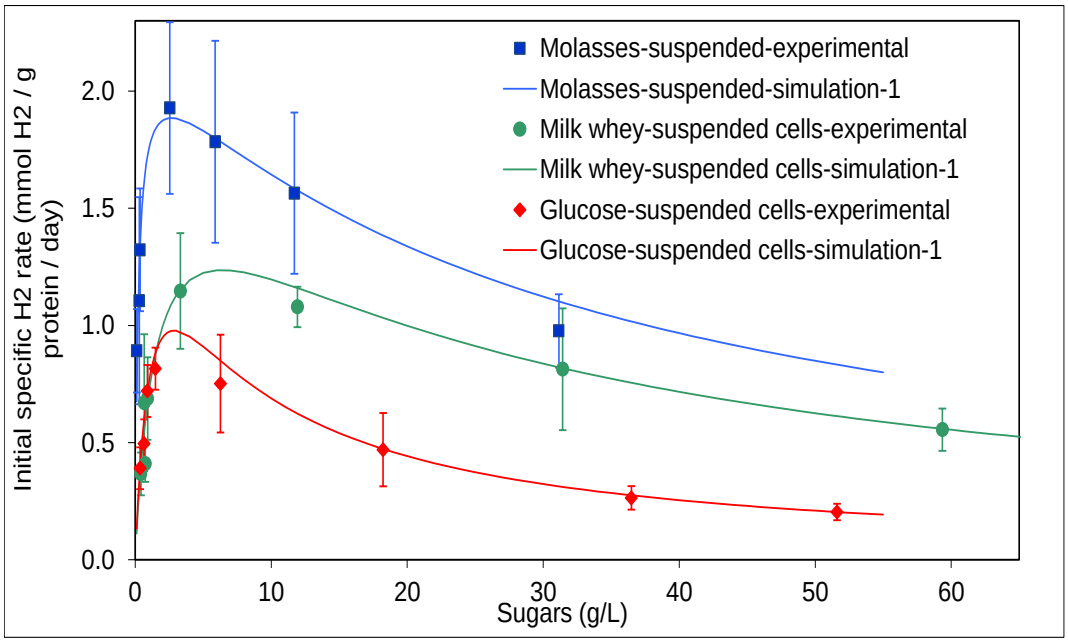

Figure 4.34 - Comparison among the three substrates with suspended biomass

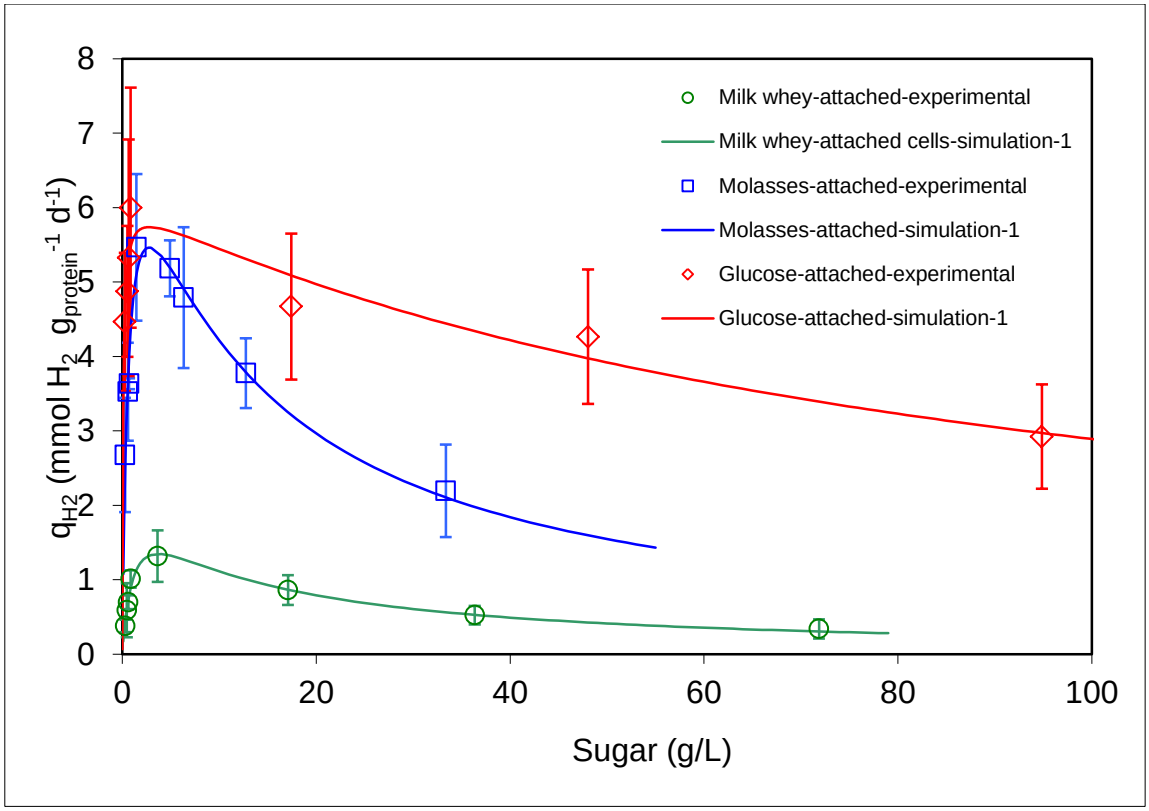

Figure 4.35 - Comparison among the three substrates immobilized biomass

In conclusion substrate inhibition was observed for all the three substrates. In the case of suspended biomass, the best results were obtained using the molasses, towards which *T. neapolitana* shows great affinity, low values of K_s, and low substrate inhibition, high K_i that becomes appreciable only at very high values of K_i.

However, even for glucose and whey substrate inhibition appears to be significant only at sugars of concentrations in the order of few *g/L*. In table 4.11 two interesting reference values of *S* are calculated for each tests: i) the substrate concentration at which the maximum H_2 specific product rate is reached (S_{max}) and ii) the value below which inhibition can be neglected, namely S^* is the value for which the inhibition term S^2/K_I is 1/10 of the (Ks+S) in the equation rate denominator. The S_{max} and S^* values in table 4.11 show that the fermentation can be carried out in a perfectly mixed bioreactor (chemostat) with unconverted substrate concentration up to 2 g/L with high specific H_2 formation rates and without a significant rate reduction due to inhibition.

The chance to use alternative substrates, like molasses and milk whey, depends also on other important parameters such as their availability, at least with an acceptable transportation cost, and the unit price. For all these reasons, even if the H_2 production rates

are not particularly high and the benefit of using immobilized biomass is relatively low, it is much more interesting to carry out the scale-up process using the whey instead of the molasses: it is cheaper, readily available and represents one of the most problematic industrial residues in the area of Emilia Romagna.

4.10 TESTS IN BIOREACTOR

The feasibility of H_2 production from milk whey as the growth substrate was evaluated some tests performed with a 20L reactor.

Tests with 20L reactor were divided into 3 steps:

1. Optimization of the inoculum volume, using glucose as substrate;
2. Batch tests using milk whey in conditions of suspended and immobilized biomass;
3. Continuous testes under immobilized biomass conditions.

4.10.1 INFLUENCES THE VOLUME OF INOCULUM

The first tests in the 20 L bioreactor were conducted using glucose as substrate, because it is a pure matrix and allows an easier evaluation of the fermentation behavior. The first evaluation was done on the influence of volume of inoculum in the H_2 production. In the 120 mL tests volumes of inoculum of 5% v/v were used, while, for tests with 20L, a lower amount 1% v/v, was also tested.

Figure 4.36 reports profiles biomass, glucose, H_2 and CO_2 versus time for a 5% v/v inoculum and Figure 4.37 for the percentage of 1% v/v inoculum.

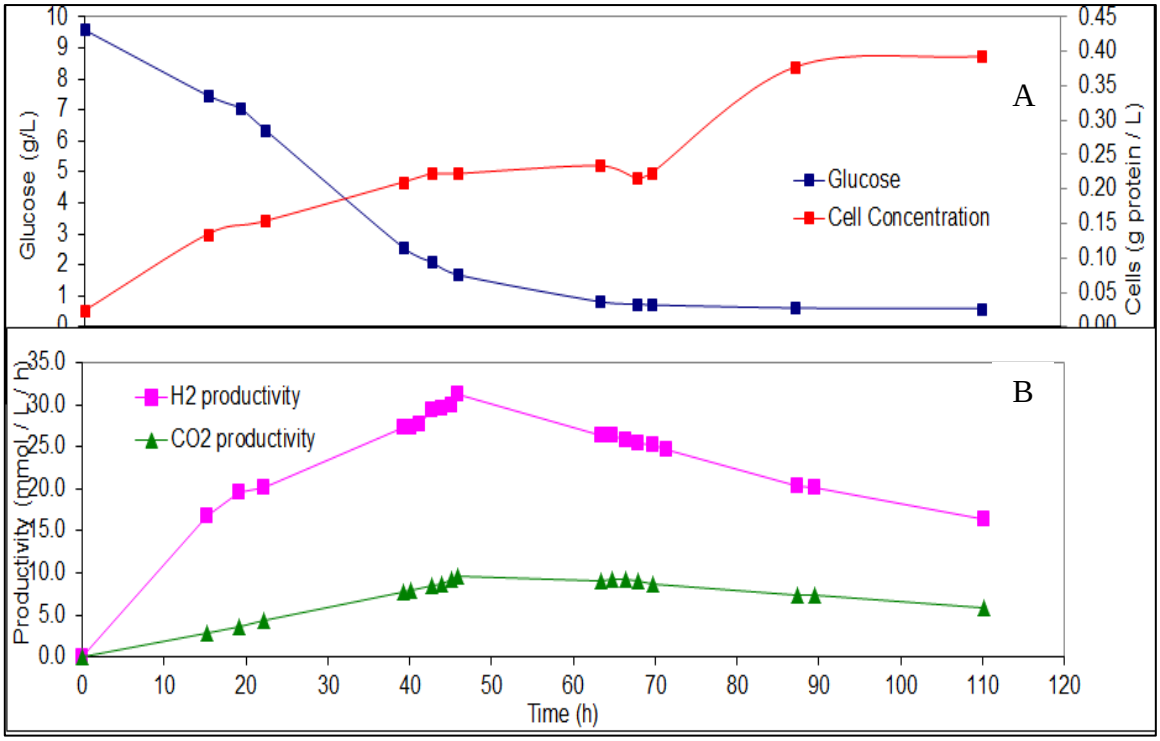

Figure 4.36 – Data of fermentation with 5% v/v inoculum. (A) Glucose and biomass concentration of (g/L), (B) mmol/L_{medium}/h H_2 and CO_2.

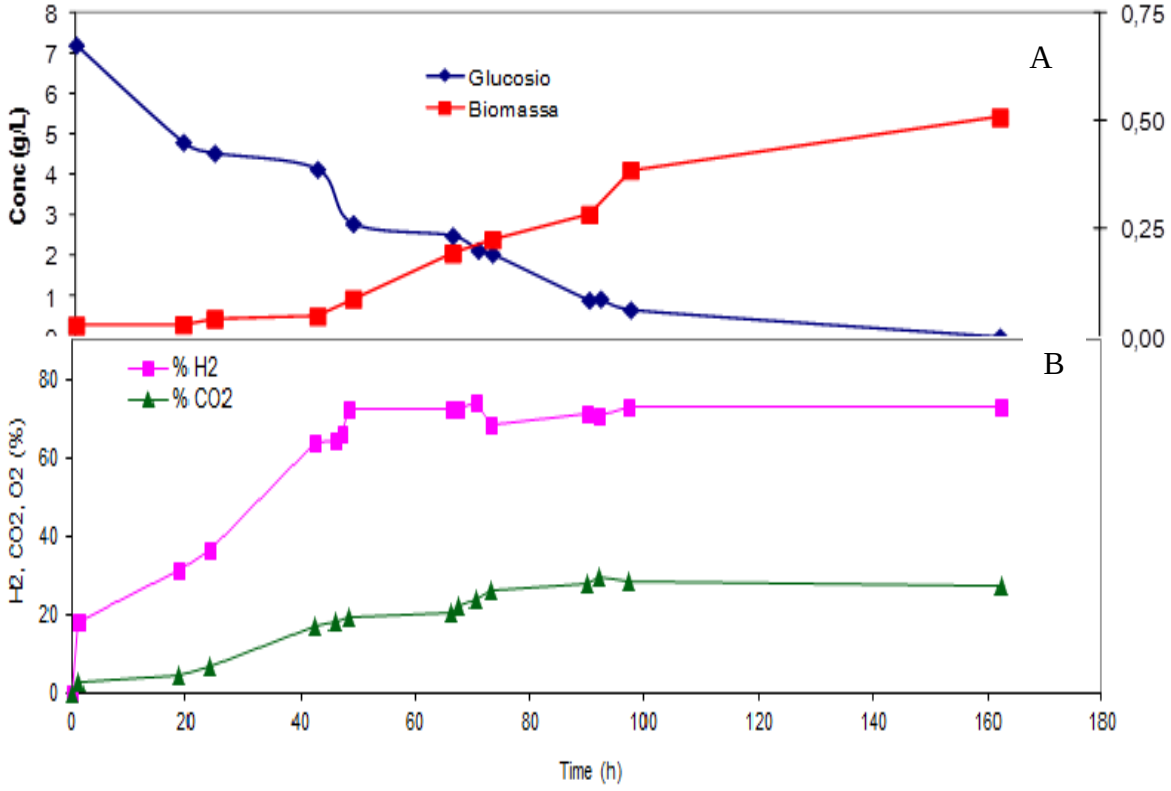

Figure 4.37 – Date of fermentation with 1% v/v inoculum. (A) Glucose and biomass concentration of (g/L), (B) mmol/L_{medium}/h H_2 and CO_2.

The analysis and comparison of the two figures show that the behavior are very similar: about the same maximum productivity was reached and about on the same batch time.

To complete the result analysis, Figure 4.38 reports the yields in H_2, biomass, acetic acid and lactic acid obtained in the two bioreactor fermentations and that obtained at small scale (120mL bottles). We can see that with the change of reactor volume there was not a significant change in the behavior of the bioreactor, the values for the 4 parameters remained very similar, the biomass in the end reaction obtained values very close to 1% and 5% inoculum.

In conclusion, as very small differences were observed working at different volumes of inoculum, it is more favorable working with 1% inoculum volume, with a reduction in costs without significant changes in the H_2, acids and biomass production.

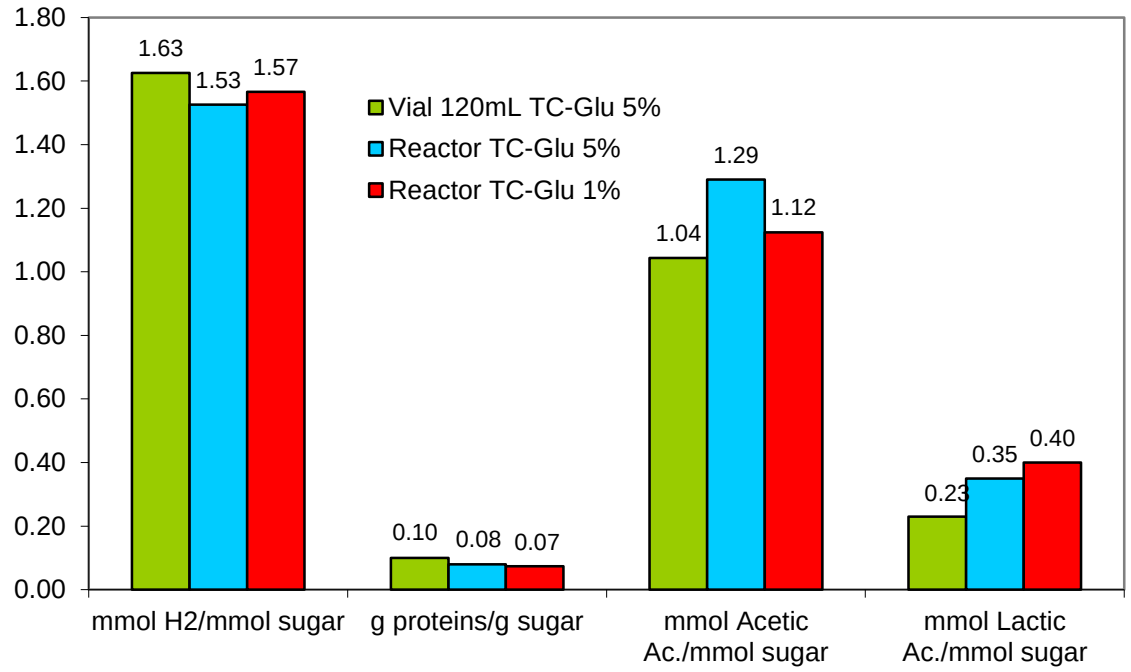

Figure 4.38 – Comparison between the parameters of 120 mL reactor with 5% v/v inoculum, to 20L with 5% v/v inoculum and 20L with 1% v/v inoculum.

4.10.2 TESTS IN BATCH WITH MILK WHEY

Milk whey 12.5 g/L was the substrate chosen for H_2 production in 20L reactor; tests were performed both with suspended and immobilized biomass.

Suspended Biomass

Figure 4.39 reports the results for suspended biomass.

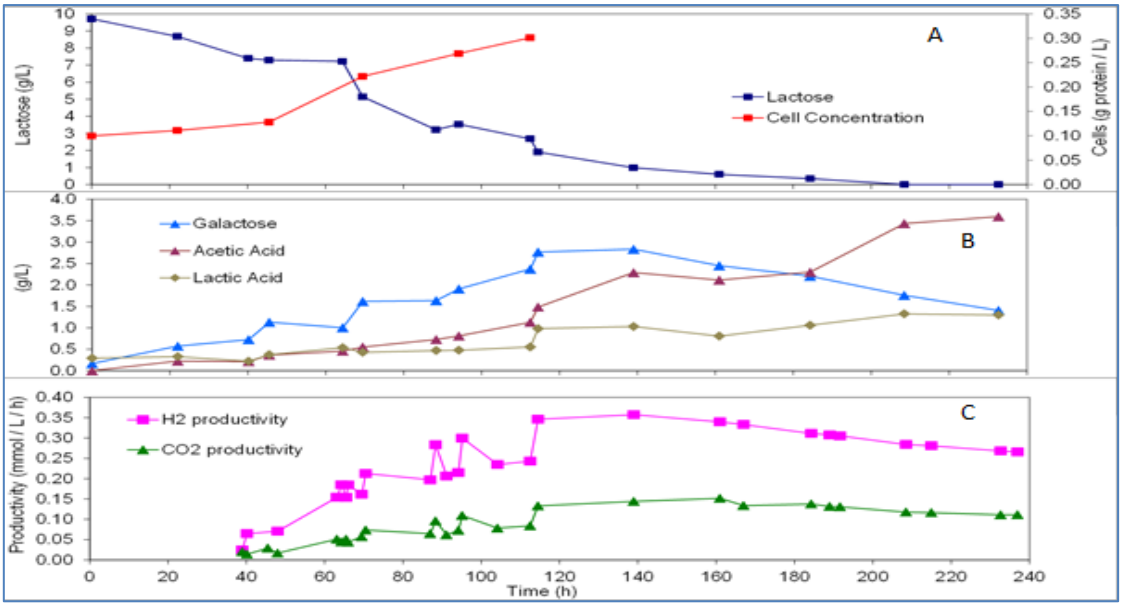

Figure 4.39 - Profiles of the products and reagents for tests with suspended biomass.

The time profiles of H_2, CO_2, sugars and acids were as expected: the hydrolysis of 1 mol of lactose produces 1 mol of glucose and 1 mol of galactose. The former is readily transformed in biomass, H_2, CO_2 and organic acids with a reaction pattern similar to that observed in the tests with glucose as substrate. On the contrary, the galactose accumulates and is only marginally metabolized. It is noteworthy that the H_2 productivity is quite lower than that obtained with glucose (1% inoculum). This low yield was probably due to the fact that only half of the initial sugar mass is available for H_2 production, that corresponding to the glucose formed.

The galactose formation and accumulation is an important scientifically aspect and describes a particular behavior of *T. neapolitana.* Diauxica growth phenomena have already been described [65] but in medium containing simultaneously glucose and lactose. In our case *T. neapolitana* is, exposed to only lactose and consumes both the two monomers obtained by the action of β-galactosidase; the glucose consumption is favored and the galactose accumulates in the medium to about t = 120h (Figure 3.39). This preference of Thermotoga towards glucose was observed up to a certain concentration of lactose; when the residual concentration of lactose goes under about 2 g/L, galactose begins to be consumed. Concerning the metabolic pathway of the *T. neapolitana* that converts the galactose into H_2 and acids, the biochemical diagram presented in the KEGG database (Kyoto Encyclopedia of Genes and Genomes) highlights the absence of the gene coding for the enzyme phosphoglucomutase (EC No. 5.4.2.2). This enzyme would catalyze the following reaction

that converts glucose α-D-glucose 1-phosphate produced by galactose into D-glucose 6-phosphate which in turn may thus enter into glycolysis.

Immobilized biomass

Tests with immobilized biomass in batch conditions were conducted to accomplish the first goal of this research work: H_2 production though immobilized biomass in a continuous bioreactor. The tests were performed as described in Materials and Methods, using (*Biomax*®) as the carrier. Figure 4.40 reports the concentrations time profiles for this test.

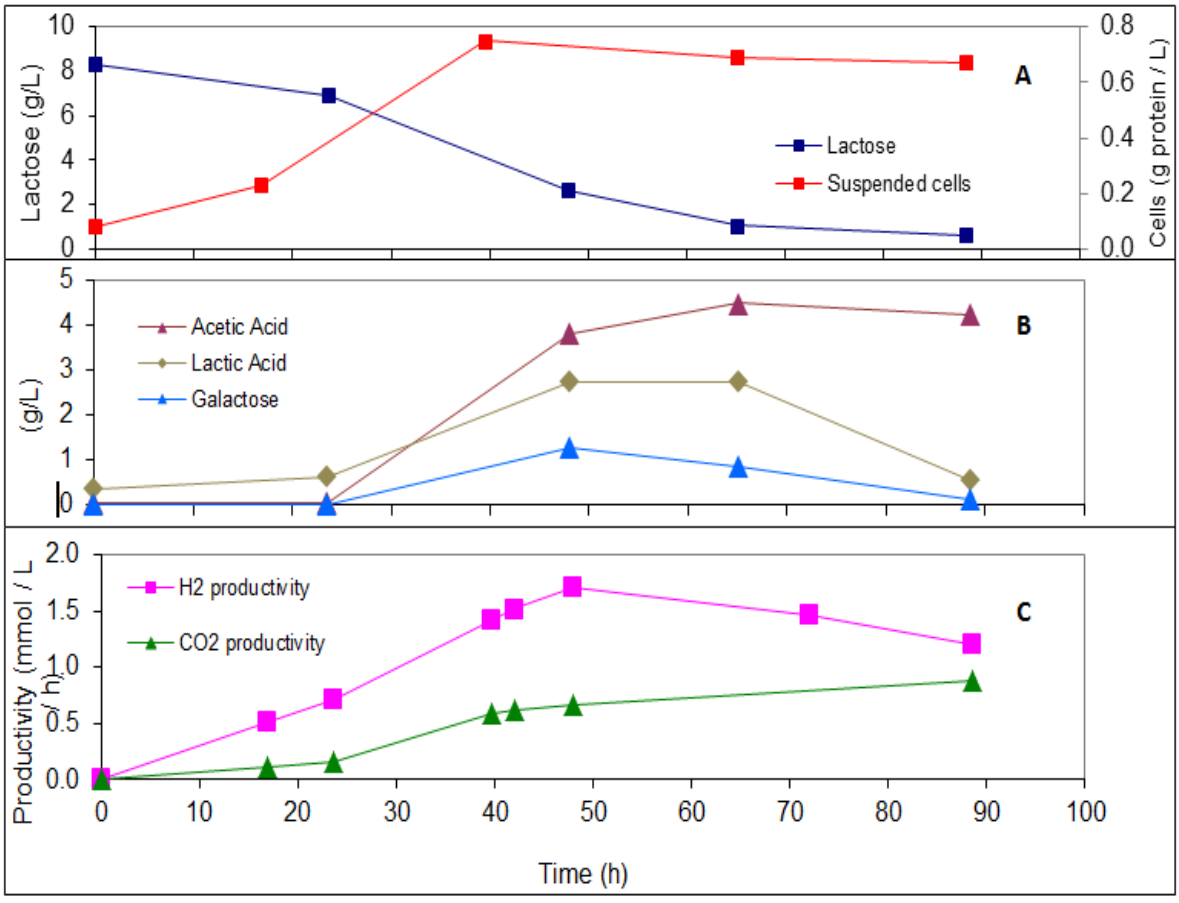

Figure 4.40 - Profiles of the products and reagents for testing with immobilized biomass.

It is to ne noted that the biomass curve in Figure 4.40 is only the suspended biomass which grows during the test due to the detachment of the immobilized biomass. It was not possible to show the values of the amounts of the immobilized biomass during the test, since for the calculation it would have been necessary to open the reactor, that was not possible during the test, this calculation was only possible once at end of the test is finished.

A noticeable results obtained working with immobilized biomass is a remarkable reduction in the batch time: with immobilized biomass 90 hours of fermentation were enough to completely consume all the sugars, while in test with suspended biomass 240 hours were required and a residual galactose concentration of 1 g/L is still unreacted.

For test with immobilized biomass in about 50 hours of fermentation we measured the highest H_2 productivity 1.75 mmol $H_2L_{medium}^{-1}$ h^{-1}; with suspended biomass the maximum was much lower and was reached after 120-140h. a additional result is that using immobilized biomass galactose was completely consumed by *T. neapolitana*.

In conclusion, the tests with immobilized biomass were very satisfactory and promising. We did not experience any problem during the fermentation of milk whey, the H_2production was higher than that with suspended biomass and the conversion time was much faster.

4.10.3 TESTS CONTINUOUS WITH IMMOBILIZED BIOMASS

The tests carried out in continuous reactor with immobilized biomass were aimed at finding the dilution rate that maximize the H_2 productivity in conditions closer to a real industrial scale. 7 different residence times were tested (Table 4.12). The initial lactose concentrations in the tests were 12.5 g/L. The hydraulic residence time is defined as the reciprocal of the dilution rate (D) that is the volumetric flow rate/medium volume ratio. In a conventional suspended biomass chemostat the productivity has a maximum for D values that are generally very near to critical dilution rate (D_C), that is the value beyond which the negative outlet convection term prevails in the mass balance over the positive biomass formation rate and no stationary state is possible. Figure 4.40 shows typical trends of biomass (X), substrate (S) and product (P) concentrations and product productivity (D_P) plotted versus the dilution rate (D) in the case of a dissolved growth related product.

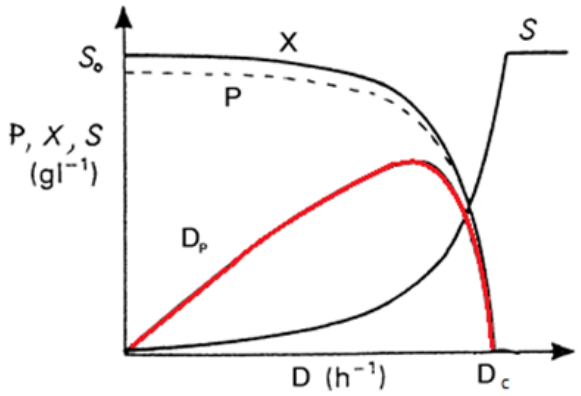

Figure 4.41: (P) Product, (X) Biomass and (S) Substrate concentrations versus increasing dilution rate (D) during a continuous culture in a chemostat.

Some change in the shape of the productivity curve versus D are expected in our conditions as the product is a gas, substrate inhibition is present and immobilized biomass is used. Anyway a similar trend with a maximum is expected and the test we carried out were aimed at identifying the optimum D value for which the maximum H_2 productivity is achieved.

Table 4.12 – Date of tests continuous.

Initial sugar (g/L)	Flow rate (L/h)	Time retention (h)	D (d^{-1})
10	0.20	70	0.343
10	0.30	48	0.510
10	0.40	35	0.673
10	0.50	28	0.857
10	0.60	24	1.024
10	0.72	20	1.236
10	0.90	15	1.543
10	1.10	13	1.886

Figure 4.42 reports the results for biogas flow rate versus D.

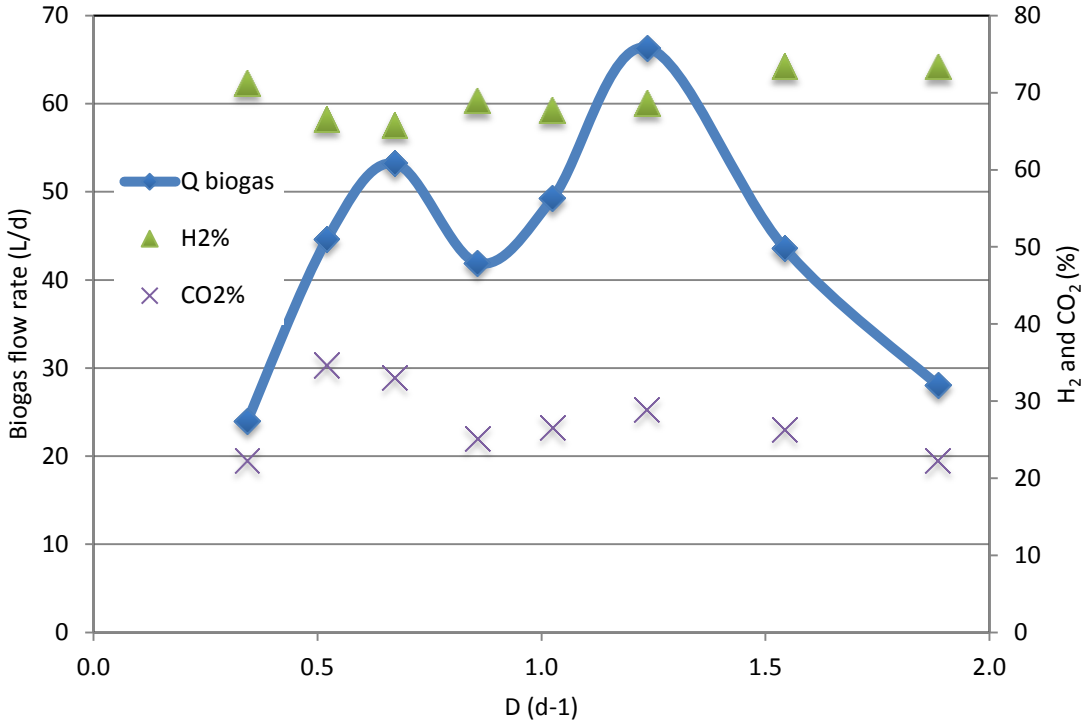

Figure 4.42- Data continuous test, biogas flow rate, H_2 and CO_2 % versus D.

In Figure 4.41 the profiles of the biogas production, H_2 and CO_2 concentrations (%) are plotted versus *D*. We can see that, even if with some consistency problems between the data, the curve of biogas productivity has a maximum in the 1-1.2 d^{-1} range. The maximum measured biogas production is 66 L/d and was achieved in the test at a dilution rate of 1.24 d^{-1}. The percentage of CO_2 and H_2 are almost constant for different D: about 70% for H_2 and 30% for CO_2.

Figure 4.43 reports the profiles of H_2 and CO_2 productivity and production.

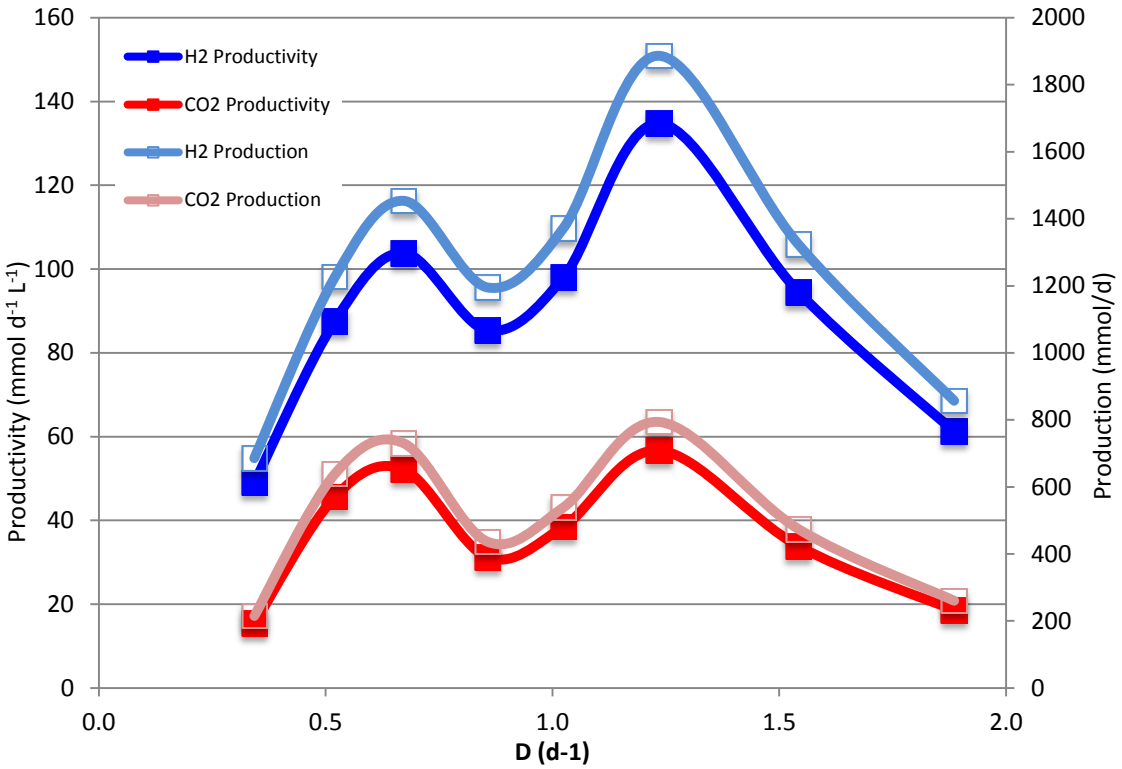

Figure 4.43- Data continuous test, productivity H_2 and CO_2 % versus D.

In Figure 4.44 the profiles for sugars consumed during the different tests are shown. Finally Figure 4.45 shows the concentrations of the acetic and lactic acids produced during the different tests.

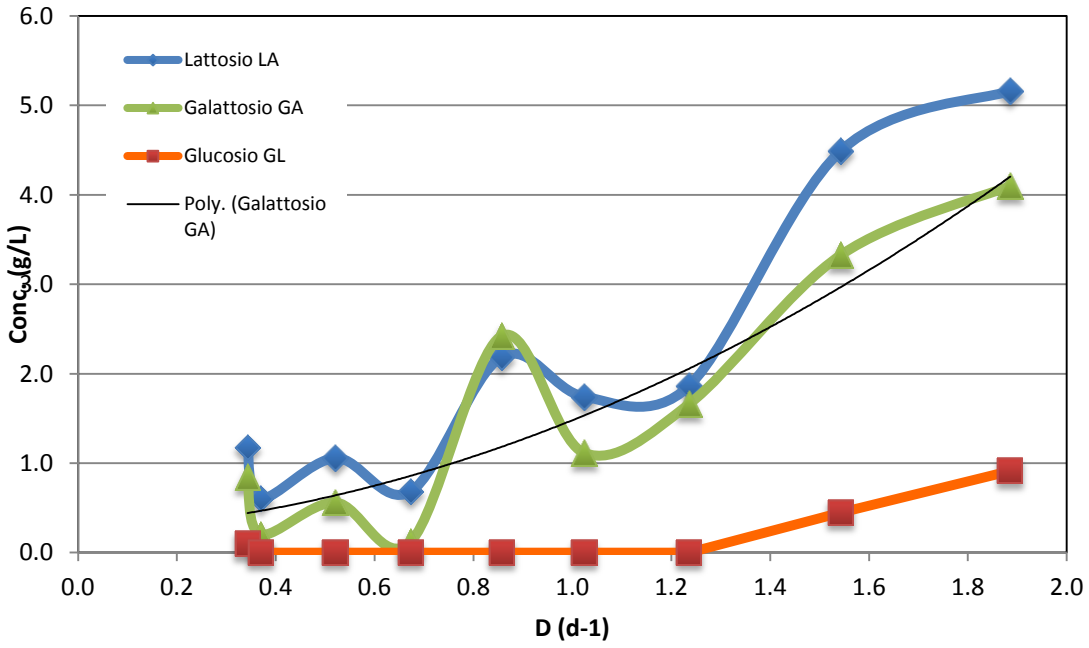

Figure 4.44- Data continuous test, concentrations to sugar versus D.

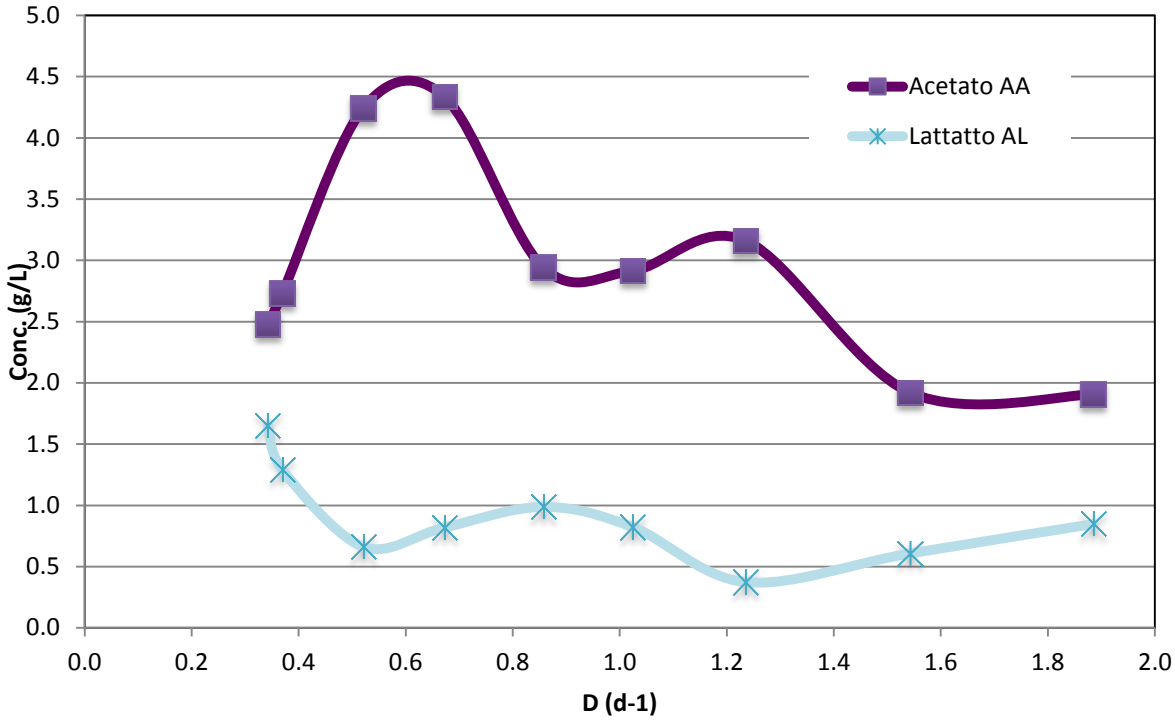

Figure 4.45- Data continuous test, concentrations to acids versus D.

We can see that increasing the dilution rate: the residence time decreases, the concentration of sugars inside the reactor increases, lactose conversion and galactose concentration decrease and a buildup of glucose is observed for *D* higher than 1.24 d^{-1}. Conversely the acids concentration decrease increasing D as they are final products of the fermentation.

In the Table 4.13 a summary of the continuous test results is reported.

Table 4.13- Summary of the continuous test.

Volumetric flow (L/h)	**Residence time τ (h)**	**D (d^{-1})**	**Q biogas (L/d)**	**H_2%**	**CO_2%**	**Sugar conversion %**
0.22	70	0.370	24	71	22	79
0.3	47	0.510	45	66	35	87
0.4	36	0.670	53	65	33	93
0.5	28	0.857	42	69	25	61
0.6	23	1.024	49	68	27	72
0.72	19	1.236	66	69	29	65
0.9	16	1.543	44	73	26	39
1.1	12	1.886	28	73	22	33

It should be noted that the maximum productivity measured in the continuous test is 5.6 *mmol* L^{-1} h^{-1}, about 3 time higher than the corresponding values obtained in the batch test with immobilized biomass 1.75 *mmol* L^{-1} h^{-1}, and higher than the 1.3 *mmol* L^{-1} h^{-1} reached with glucose with suspended biomass at a slightly lower initial sugar concentration (10 g/L of glucose instead of about 12 g/L of lactose). It should also be noted that this high H_2 production rate is reached notwithstanding galactose is only partly utilized; galactose is potentially half of the sugar mass fed, the rest being glucose produced by lactose hydrolysis.

This noticeable performance can be ascribed to the choice to work with immobilized biomass and to the efficacy of the immobilization method used in the bioreactor. Moreover the maximum productivity operating conditions, the unconverted lactose, about 2 g/L, is enough low to avoid a strong substrate inhibition.

In the Table 4.14 reports the yields for tests with 20L bioreactor, for glucose and milk whey. The maximum theoretical yield is 4 in the case of glucose or another monosaccharide and 8 for disaccharides such as lactose. Analysis of table 4.14 shows that a higher H_2 yield was obtained in continuous tests with respect to the batch test at the expenses of lactic acid yield.

In conclusion, the performances obtained in the continuous bioreactor tests were very satisfactory indeed and promising for the development of a continuous process with immobilized biomass

Table 4.14 – summarized for test with 20L bioreactor.

Test	Conditions cells	mmol H_2/mmol sugar consumed	mmol acetic ac./ mmol sugar consumed	mmol lactic ac./mmol sugar consumed
Glucose 7.5 g/L batch	Suspended	1.6	1.1	0.40
Milk whey 12.5g/L batch	Suspended	0.6	1.5	0.42
Milk whey 12.5 g/L batch	Immobilized	3.6	2.8	1.3
Milk whey 12.5 g/L continuous	Immobilized	4.7	2.3	0.1

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, different hyperthermophilic microorganisms belonging to the *Thermotoga* genus have been characterized for fermentative hydrogen production in batch and continuous cultures using food industry wastes as growth substrate. Preliminary tests suggested acidic pH inhibition mechanism on both hydrogen production and microbial growth of *T. neapolitana*, selected as model organism. Inorganic and organic pH buffers were, therefore, tested in order to prevent pH inhibition and allow both high hydrogen volumetric productivities and efficient substrate to hydrogen conversion yields. The best pH buffer capacity in terms of final pH values and the best hydrogen productivity was achieved in *T. neapolitana* batch cultures (120 mL) supplemented with HEPES, with an optimal concentration of 100 mM. Furthermore, a range of HEPES concentration between 50 mM and 200 mM maintained analogous pH values and comparable hydrogen volumetric productivities. Thus, the aim to find an efficient buffer to carry out further experiments in not inhibiting pH conditions was achieved.

The four hyperthermophilic *Thermotoga* strains selected for this project were *T. neapolitana, T. maritima, T. naphtophila,* and *T. petrophila*. Hydrogen production by each microorganism growing on glucose and food industry wastes (e.g. sugar beet molasses and milk whey)was investigated, both in suspended and immobilized cell cultures. For tests with immobilized biomass, a commercial biofilm carrier, *Biomax*®, but tests for the choice of the best support were made in following step.

To date, no studies investigated hydrogen production by either *T. naphtophila* and *T. petrophila* batch cultures or *Thermotoga* strains from milk whey or sugar beet molasses. Hydrogen production by *T. naphtophila* and *T. petrophila* were obtained on modATCC 1977 medium supplemented with glucose 7.5 g/L. Moreover, the feasibility of hydrogen production process by using molasses and whey as carbohydrates sources was demonstrated for all the microorganisms tested, both in suspended cell and immobilized cell tests. Hydrogen productivities were in the same order of magnitude of values reported in previous studies describing cultures of *Thermotoga* growing on glucose as main carbon source. Thus, our work demonstrated that milk whey and molasses carbohydrates can be efficiently converted into hydrogen by hyperthermophilic *Thermotoga* strains.

Suspended cell tests confirmed high conversion yields of glucose into hydrogen by *Thermotoga* strains. Interestingly, milk whey and molasses carbohydrates were also efficiently converted into hydrogen and values obtained were in the same order of magnitude of the theoretical yield of 4 moles of H_2 per mole of glucose consumed. Moreover, average hydrogen production rates of every microorganism tested was comparable with data obtained in previous studies describing cultures of *Thermotoga* growing on media supplemented with glucose as main carbon source. In particular, *T. neapolitana* achieved the highest volumetric production rate by growing on all the substrates under analysis and it showed the conversion yields of substrate into hydrogen yields next to the theoretical maximum. Moreover, *T. neapolitana* volumetric production rates from glucose, molasses and milk whey (1.92, 1.70, 0.94 mmol $H_2L^{-1}h^{-1}$, respectively) were equivalent or higher than the values reported in literature.

Hydrogen production by *Thermotoga* immobilized cells from glucose, milk whey and sugar beet molasses was performed. All the microorganisms succeeded to grow and to form a biofilm on *Biomax*®. High values of immobilized biomass concentration were achieved. *T. neapolitana* obtained the most efficient hydrogen production in terms of specific and volumetric production rates. Moreover, also in the case of suspended biomass, *T. neapolitana* cultures grown on glucose, whey and molasses showed the highest volumetric production rates among the four microorganisms tested. Hydrogen production rates achieved by *T. neapolitana* were even higher than any data reported in literature. Thus, bioconversion of organic wastes by *T. neapolitana* suspended and immobilized cell was optimized obtaining a very efficient process.

For immobilized biomass the molasses showed a better performance. Moreover, analyzing *T. neapolitana* production rates from glucose, molasses and milk whey (1.4, 1.8, 1.2 mmol $H_2L^{-1}h^{-1}$, respectively) we have seen that for glucose the productivity was slightly lower than that obtained with suspended biomass.

Further experiments were carried out to find more economical media based on organic wastes to be employed for the growth of *T. neapolitana*, which was chosen among the other *Thermotoga* strains according to previous results. Firstly, sterilization was demonstrated to be unnecessary, because no microbial growth at high temperatures was detected in the medium. This data confirmed previous reports and it is economically important in order to reduce production costs. Main components of whey and molasses growth media were omitted. High production rates and conversion yields of substrate into hydrogen by *T. neapolitana* were

maintained in milk whey minimal medium, and molasses minimal medium. Our experiments indicated that the media with either molasses or milk whey to be utilized for *T. neapolitana* growth required only the addition of a nitrogen source, a phosphate source and cysteine as reducing agent, which was demonstrated to be essential. With both molasses and milk whey, the progressive simplification of the *T. neapolitana* growth medium allowed the attainment of a 90% and 60% reduction of medium cost respectively, without significant losses of process performance.

To date, no studies investigated hydrogen production by *Thermotoga* immobilized cells. Four solid porous carriers were tested in order to examine *T. neapolitana* immobilized biomass formation and hydrogen production. *T. neapolitana* was able to form biofilm by growing on modATCC 1977 medium on all the carriers under analysis and to produce hydrogen. *T. neapolitana* achieved the highest immobilized biomass concentration, and the highest volumetric and specific hydrogen production rates were obtained by using *Biomax*® as biofilm carrier. According to this result, *Biomax*® was chosen for subsequent immobilized cell experiments.

The tests to optimize the pH for the H_2 production showed that the optimal pH is in the range 7.6-8.5. For all tests we used pH 8.5.

Tests were conducted to assess the sensitivity of *T. neapolitana* to the presence of O_2 in the head space, being the *T. neapolitana* an anaerobic bacterium. Tests were carried out varying the concentrations of O_2in the head space from 0 to 1%. The tests were performed only for the substrate milk whey, which is the substrate chosen for the 20L bioreactor tests. We concluded that H_2 production with both suspended and immobilized biomass was not inhibited by the presence of O_2.

Hydrogen inhibition on Thermotoga species was investigated for the three substrates, glucose, molasses and milk whey, and two biomass conditions suspended and immobilized. Tests shown that for the three substrates no product inhibition occurs up to about 60% of H_2 in the head space.

Kinetic studies were finally performed to investigate a suitable lumped equation for the hydrogen production rate. An acompetitive substrate inhibition kinetic model was successfully used (Andrews model).The results showed that *T. neapolitana* is characterized by low substrate constant (K_S) values and high substrate inhibition constant (K_I) values for each one of the substrates tested. These data confirm the possibility to work in bioreactor with moderate concentration of substrate without having excessive substrate inhibition.

Moreover, *T. neapolitana* cells can produce hydrogen at the maximum specific rate with low concentration of substrate.

In order to study the feasibility of an immobilized biomass continuous process, some tests were carried out in 20 L bioreactor. The process performance was satisfactory; the H_2 productivity values obtained with the 20L reactor were higher than those obtained with the small scale fermentation (120 mL). The tests were performed at different dilution rate and the value corresponding to the maximum H_2 productivity was determined. For the tests an innovative bioreactor was designed and developed.

In conclusion, our work proved the feasibility of an efficient fermentative hydrogen production process from economic media by *Thermotoga* using immobilized cells.

6. BIBLIOGRAPHIC

1. Vicente, G.; Colteron, A.; Martinez, J.; Aracil, J. Application of the factorial design of experiments and response surface meyhodology to optimize biodiesel production. Industrial Crops and Products.n. 8, p. 28-35, 1998.
2. Hallenbeck, P.C. and D. Ghosh, Advances in fermentative biohydrogen production: the way forward?Trends Biotechnol, 2009. 27(5): p. 287-97.
3. Hallenbeck PC and Benemann JR, Biological hydrogen production: fundamentals and limiting processes. Int J Hydrogen Energy 27:1185–1193 (2002).
4. Das DandVeziroglu TN,Hydrogen production by biological processes: a survey of literature.Int J Hydrogen Energy 26:13–28 (2001).
5. Levin DB, Pitt L and Love M, Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 29:173–185 (2004)
6. Kersti Johansson, Karin Liljequist, Lars Ohlander, Kjell Aleklett, Agriculture as Provider of Both Food and Fuel. AMBIO (2010) 39:91–99
7. Arnold L. Demain, Biosolutions to the energy problem. J Ind Microbiol Biotechnol (2009) 36:319–332
8. Sanderson KW (2007) Are ethanol and other biofuels technolo-gies part of the answer for energy independence? Cereal Food World 52:5–7
9. Greene N, Roth R (2006) Ethanol: energy well spent. A review of corn and cellulosic energy balances in the scientiWc literature to date. Ind Biotechnol 2:36–39
10. AAVV, in Eidgenoessischen Technischen Hochschule Zurich. 2000.
11. Zuttel, A., et al., Hydrogen: the future energy carrier.Philos Transact A Math Phys Eng Sci, 2010. 368(1923): p. 3329-42.
12. Abreu, A.A., Danko, A.S., Alves, J.I. and Alves, M.M. Fermentative Bio-Hydrogen Production From Organic Wastes And Sugars. Bioenergy: Challenges and Opportunities International Conference and Exhibition on Bioenergy, April 6th – 9th 2008
13. "Biomass" New University of Lisbon. (n.d). From: http://campus.fct.unl.pt
14. Agri-food industry. (n.d). From: http://www.ipa-alimenti.it/azienda-verde/
15. Bianchi, M., F. Melino, and A. Peretto, Sistemi energetici 2 – Complementi. 2008: Pitagora Editrice Bologna.

16. Laboratory environment and marine technology, University of Azores. From: http://www.lamtec-id.com/energias/hidrogenio.php
17. The water electrolysis. From: http://www.mcphy.com/en/technologies/water-electrolysis/
18. Water Oxidation Catalysts for Bio-Inspired Photoelectrochemical Cells, Monash University.From:http://monash.edu/science/about/schools/chemistry/staff/spiccia/waterox.html
19. Hallenbeck, Patrick C. Biological hydrogen production; fundamentals and limiting processes. International Journal of Hydrogen Energy 2002, 27: 1185 – 1193
20. Tamagnini, P, Axelsson, R, Lindberg, P, Oxelfelt, F, Wünschiers, R e Lindblad, P (2002) Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiol. Mol. Biol. Rev., 66: 1-20.
21. Tamagnini, P, Costa, J-L, Almeida, L, Oliveira, M-J, Salema, R e Lindblad, P (2000) Diversity of cyanobacterial hydrogenases, a molecular approach. Curr. Microbiol., 40: 356-361.
22. Ghirardi, M.L., et al., Microalgae: a green source of renewable H_2.Trends Biotechnol, 2000. 18(12): p. 506-11.
23. Cellular processes. From:http://library.thinkquest.org/C004535/glycolysis.html.
24. Hallenbeck, P.C., *Fermentative hydrogen production: Principles, progress, and prognosis.* International Journal of Hydrogen Energy, 2009. 34: p. 7379-7389.
25. Vardar-Schara, G.; Maeda T.; Wood, T. K.. Metabolically engineered bactéria for producing hydrogen via fermentation. Microbial Biotechnology, v. 1, n. 2, p. 107-125, 2008.
26. Vanfossen, A.L., et al., *Polysaccharide degradation and synthesis by extremely thermophilic anaerobes.* Ann N Y Acad Sci, 2008. 1125: p. 322-37.
27. Van Ooteghem, S.A., et al., *H(2) production and carbon utilization by Thermotoga neapolitana under anaerobic and microaerobic growth conditions.* Biotechnol Lett, 2004. 26(15): p. 1223-32.
28. Chou, C.J., et al., *Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels.* Metab Eng, 2008. 10(6): p. 394-404.
29. Thauer, R.K., K. Jungermann, and K. Decker, *Energy conservation in chemotrophic anaerobic bacteria.* Bacteriol Rev, 1977. 41(1): p. 100-80.

30. Deguchi, S., K. Tsujii, and K. Horikoshi,*Cooking cellulose in hot and compressed water.* Chem Commun (Camb), 2006(31): p. 3293-5.
31. Yu, J.S., et al., *Liposome-mediated DNA uptake and transient expression in Thermotoga.* Extremophiles, 2001. 5(1): p. 53-60.
32. Jiang, Y., et al., *A highly efficient method for liquid and solid cultivation of the anaerobic hyperthermophilic eubacterium Thermotoga maritima.* FEMS Microbiol Lett, 2006. 259(2): p. 254-9.
33. Tsudome, M., et al., *Versatile solidified nanofibrous cellulose-containing media for growth of extremophiles.* Appl Environ Microbiol, 2009. 75(13): p. 4616-9.
34. Stetter, K.O., *History of discovery of the first hyperthermophiles.* Extremophiles, 2006. 10(5): p. 357-62.
35. Verhaart, M.R., et al., *Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal.* Environ Technol, 2010. 31(8-9): p. 993-1003.
36. Takahata, Y., et al., *Thermotoga petrophila sp. nov. and Thermotoga naphthophila sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan.* Int J Syst Evol Microbiol, 2001. 51(Pt 5): p. 1901-9.
37. Conners, S.B., et al., *Microbial biochemistry, physiology, and biotechnology of hyperthermophilic Thermotoga species.* FEMS Microbiol Rev, 2006. 30(6): p. 872-905.
38. Worning, P., et al., *Structural analysis of DNA sequence: evidence for lateral gene transfer in Thermotoga maritima.* Nucleic Acids Res, 2000. 28(3): p. 706-9.
39. Zhaxybayeva, O., et al., *On the chimeric nature, thermophilic origin, and phylogenetic placement of the Thermotogales.* Proc Natl Acad Sci U S A, 2009. 106(14): p. 5865-70.
40. Lee, D., et al., *WeGAS: a web-based microbial genome annotation system.* Biosci Biotechnol Biochem, 2009. 73(1): p. 213-6.
41. Nelson, K.E., et al., *Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of Thermotoga maritima.* Nature, 1999. 399(6734): p. 323-9.
42. d'Ippolito, G.D., L.; Vella, F.M.; Romano, I.; Gambacorta, A., Cutignano, A.; Fontana, A., Hydrogen metabolism in the extremophile Thermotoga neapolitana.International Journal of Hydrogen Energy, 2010. 35: p. 2290-2295.

43. Schut, G.J. and M.W. Adams, *The iron-hydrogenase of Thermotoga maritima utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production.* J Bacteriol, 2009. 191(13): p. 4451-7.
44. Vargas, M. and K.M. Noll, *Catabolite repression in the hyperthermophilic bacterium Thermotoga neapolitana is independent of cAMP.* Microbiology, 1996. 142 (Pt 1): p. 139-44.
45. Kadar, Z., et al., *Hydrogen production from paper sludge hydrolysate.* Appl Biochem Biotechnol, 2003. 105 -108: p. 557-66.
46. Eriksen, N.T., et al., *H(2) synthesis from pentoses and biomass in Thermotoga spp.* Biotechnol Lett, 2011. 33(2): p. 293-300.
47. Munro, S.A., S.H. Zinder, and L.P. Walker, *The fermentation stoichiometry of Thermotoga neapolitana and influence of temperature, oxygen, and pH on hydrogen production.* Biotechnol Prog, 2009. 25(4): p. 1035-42.
48. de Vrije, T., et al., *Efficient hydrogen production from the lignocellulosic energy crop Miscanthus by the extreme thermophilic bacteria Caldicellulosiruptor saccharolyticus and Thermotoga neapolitana.* Biotechnol Biofuels, 2009. 2(1): p. 12.
49. Van Ooteghem, S.A., S.K. Beer, and P.C. Yue, *Hydrogen production by the thermophilic bacterium Thermotoga neapolitana.* Appl Biochem Biotechnol, 2002. 98-100: p. 177-89.
50. Eriksen, N.T., T.M. Nielsen, and N. Iversen, *Hydrogen production in anaerobic and microaerobic Thermotoga neapolitana.* Biotechnol Lett, 2008. 30(1): p. 103-9.
51. Nguyen, D., et al., *Hydrogen production by the hyperthermophilic eubacterium, Thermotoga neapolitana, using cellulose pretreated by ionic liquid.* International Journal of Hydrogen Energy, 2008. 33: p. 5161-5168.
52. Nguyen, T.A., et al., *Hydrogen production of the hyperthermophilic eubacterium, Thermotoga neapolitana under N2 sparging condition.* Bioresour Technol, 2010. 101 Suppl 1: p. S38-41.
53. Childers, S.E., M. Vargas, and K.M. Noll, *Improved Methods for Cultivation of the Extremely Thermophilic Bacterium Thermotoga neapolitana.* Appl Environ Microbiol, 1992. 58(12): p. 3949-53.

54. Wang, J. and W. Wan, *Factors influencing fermentative hydrogen production: A review.* International Journal of Hydrogen Energy, 2009. 34: p. 799-811.
55. Lin, C. Y.; Chang, R. C. Hydrogen production during the anaerobic acidogenic conversion of glucose. J Chem Technol Biotechnol, v.74, n.6, p. 498-500, 1999.
56. Noike, T.; Mizuno, O. Hydrogen fermentation of organic municipal waste. Waste Sci Technol., v.42, n12, p. 155-162, 2000.
57. Brenner, K., L. You, and F.H. Arnold, *Engineering microbial consortia: a new frontier in synthetic biology.* Trends Biotechnol, 2008. 26(9): p. 483-9.
58. Ueno, Y., H. Fukui, and M. Goto, *Operation of a two-stage fermentation process producing hydrogen and methane from organic waste.* Environ Sci Technol, 2007. 41(4): p. 1413-9.
59. Davey, M. E. e O'toole, G.A. (2000) Microbial Biofilms: from Ecology to Molecular Genetics. Microbiology and Molecular Biology Reviews, 64, 847-867.
60. Elvers, T. K., and Lappin-Scott, M. H., (2004) Biofilms and biofouling. The Desk Encyclopedia of Microbiology. 12. P. 161-167.
61. Marshall, K. C., (1976) Interfaces in microbial ecology. Harvard University Press, Cambridge.
62. Biofilm basics, The website for the Center for Biofilm Engineering, U. of Montana, http://www.erc.montana.edu/
63. Flemming, H. C., Wingender, J., (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633.
64. Schröder C, Selig M, and Schönheit P, Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritime* - involvement of the Embden-Meyerhof pathway. *Arch Microbiol* 161:460–470 (1994).
65. Vargas, M. and K.M. Noll. 1996. Catabolite repression in the hyperthermophilic bacterium *Thermotoga neapolitana* is independent of cAMP. Microbiology. 142:39.
66. Peterson GL, A simplification of the protein assay method of Lowry, et al.which ismore generally applicable.Anal Biochem83:346–356 (1977).