Hydrogen production from biomaterials by the extreme thermophile *Caldicellulosiruptor saccharolyticus*

Ph.D. Thesis

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Abbreviations:

AA: alginic acid

BGS: sugarcane bagasse

CB: cellobiose

CFU: colony forming unit

GAC: granular activated carbon

LC: liquid (suspended) culture

LMA: low melting point agarose

NADH: nicotinamide adenine dinucleotide phosphate

PER: perlite

PBS: phosphate-buffered saline (20 mM K-phosphate buffer, 160 mM NaCl, pH 7.0)

PML: pretreated maize leaves

PWS: pine wood shavings

SSC: sweet sorghum concentrate 65 ^{o}Bx

STR: Silphium tripholiatum

SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis

SSJ: sweet sorghum juice

SSP: sweet sorghum plant

TC: total carbon

TOC: total organic carbon

UML: untreated maize leaves

WST: wheat straw

YE: yeast extract

1. Overview of literature

1.1. Hydrogen biotechnology

1.1.1. General use and production of hydrogen

The popularity of hydrogen as a fuel source followed the energy crisis during the 1970s resulting from the excessive use of non-renewable fuels. With the 1990s concerns about the “greenhouse effect” a new crisis reignited interest in hydrogen as a fuel. Nowadays it is widely acknowledged that hydrogen is an attractive energy source to replace conventional fossil fuels, both from environmental and economic standpoint. When hydrogen is used as a fuel it generates no pollutants but produces water which can be recycled to make more hydrogen (Figure 1).

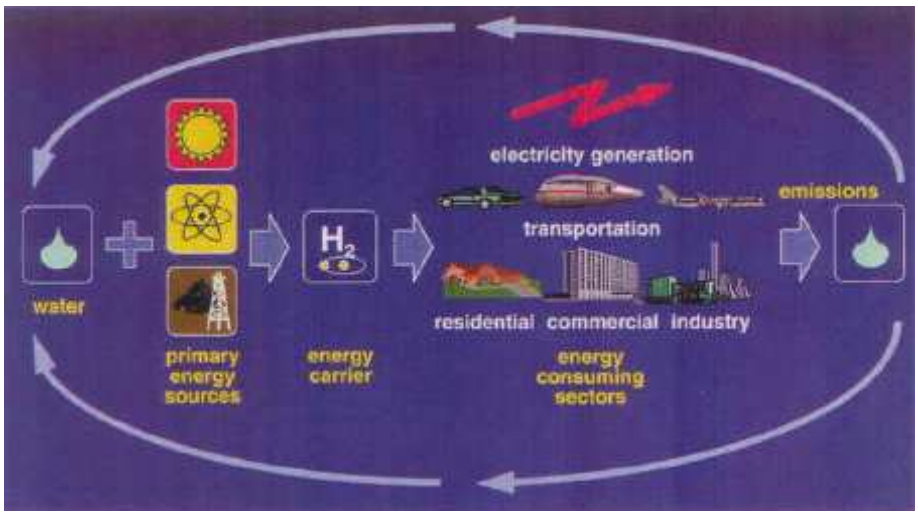


Figure 1. Hydrogen energy system. (Source: International Association of Hydrogen Energy, USA).

Apart from its use as a clean energy carrier, hydrogen can be used for various other purposes in chemical process industries. It is used as a reactant in hydrogenation process to produce lower molecular weight compounds. It can also be used to saturate compounds, crack hydrocarbons or remove sulphur and nitrogen compounds. It is a good oxygen scavenger and

can therefore be used to remove traces of oxygen to prevent oxidative corrosion in the presence of a catalyst. In the manufacturing of ammonia, methanol and synthesis gas, the use of hydrogen is well known. The future widespread use of hydrogen is likely to be in the transportation sector, where it will help reduce pollution. Vehicles can be powered with hydrogen fuel cells, which are three times more efficient than a gasoline-powered engine. As of today, all these areas of hydrogen utilization are equivalent to 3% of the energy consumption, but it is expected to grow significantly in the years to come (Nath and Das 2003).

The currently commercially used hydrogen is extracted mostly from natural gas. Nearly 90% of hydrogen is obtained by steam reformation of naphtha or natural gas. Gasification of coal and electrolysis of water are the other industrial methods for hydrogen production (Tanisho et al. 1983). However, these processes are highly energy intensive and not always environment-friendly. Moreover, the fossil-fuel (mainly petroleum) reserves of the world are depleting at an alarming rate. Given the economic uncertainties and environmental hazards of fossil fuels, working out the technical and economic feasibility of hydrogen production is becoming a major priority in the 21st century. Biotechnology, in that aspect, is uniquely poised to make a significant contribution to that effort.

1.1.2. Biohydrogen generation and the role of hydrogenases in H_2 metabolism

Molecular hydrogen produced from renewable sources (biomass, water, organic wastes) either biologically or photobiologically is called “biohydrogen”. It can be achieved by the use of two main types microorganisms, photosynthetic (photoautotrophic and photoheterotrophic) and fermentative. Reports on microbial H_2 productions have been periodically reviewed by various scientists (Pakes and Jollyman 1901; Harden 1901; Zajic et al. 1978; Adams et al. 1981; Roychowdhury et al. 1988; Sasikala et al. 1993; Wu and Mandrand 1993). The production of H_2 by different microorganisms is intimately linked to their respective energy metabolism. In aerobic microorganisms, released electrons from substrate oxidation is transferred to oxygen as the ultimate oxidant, while in anaerobic microorganisms electrons released from anaerobic catabolism use many terminal oxidants such as nitrate, sulphate, or organic compounds derived from carbohydrates as carbon source.

Hydrogen metabolism in microorganisms is carried out by metalloenzymes, namely nitrogenases and hydrogenases. Nitrogenases release H_2 as a byproduct during nitrogen fixation. Hydrogenases catalyze the simplest chemical reaction: $2H^+ + 2e^- \leftrightarrow H_2$. The reaction is reversible, and its direction depends on the redox potential of the components able to interact with the enzyme. In the presence of H_2 and an electron acceptor, it will act as a H_2 uptake enzyme; in the presence of an electron donor of low potential, it may use the protons from water as electron acceptors and release H_2. The production of H_2 is one of the specific mechanisms to dispose excess electrons through the activity of hydrogenases present in H_2 producing microorganisms. Hydrogenase activity can be measured *in vitro*, using artificial or natural electron carriers. The *in vivo* function of the hydrogenases depends on the current redox status of the cell.

Hydrogenases have various physiological roles. They have a different localization as well as a different subunit composition in the cell. The first classification of these enzymes was based on the identity of specific electron donors and acceptors, namely, NAD. Until 2004, hydrogenases were classified according to the metals at their active sites. Three main classes were recognized: iron-only ([FeFe] hydrogenases), nickel-iron ([NiFe] hydrogenases), and "metal-free" hydrogenases. In 2004, Lyon et al. showed that the metal-free hydrogenases in fact contain iron. Thus, those enzymes previously called "metal-free" are now named "iron-sulfur-cluster-free" hydrogenases, since they contain no inorganic sulfide in contrast to the Fe-only enzymes. In some [NiFe] hydrogenases, one of the Ni-bound cysteine residues is replaced by selenocysteine. On the basis of sequence similarity, however, the [NiFe] and [NiFeSe] hydrogenases belong to the same superfamily.

The [NiFe] hydrogenases are heterodimeric proteins consisting of small (about 30 kDa) and large (about 60 kDa) subunits. The small subunit contains three iron-sulfur clusters while the large subunit contains a nickel-iron centre. Periplasmic, cytoplasmic, and cytoplasmic membrane-bound hydrogenases have been found. The [NiFe] hydrogenases, when isolated, are found to catalyse both H_2 evolution and uptake, with low-potential multihaem cytochromes such as cytochrome c_3 acting as either electron donors or acceptors, depending on their oxidation state.

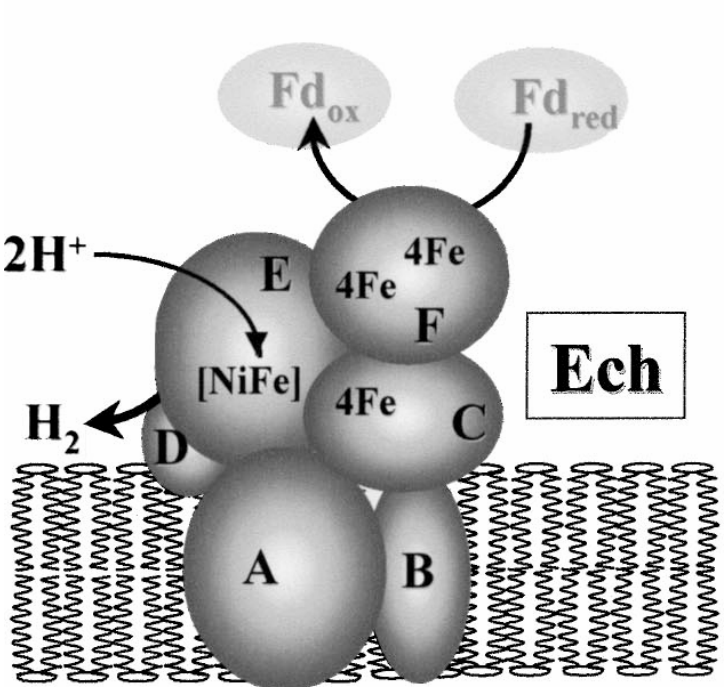


Figure 2. A scheme of [NiFe] hydrogenase in *Methanosarcina barkeri* (Hedderich 2004).

The hydrogenases containing Fe-S clusters and no other metal than iron are called Fe-hydrogenases. Three families of Fe-hydrogenases are recognized:

I. Cytoplasmic, soluble, monomeric Fe-hydrogenases, found in strict anaerobes such as *Clostridium pasteurianum* and *Megasphaera elsdenii*. They are extremely sensitive to inactivation by dioxygen (O_2) and catalyse both H_2 evolution and uptake;

II. Periplasmic, heterodimeric Fe-hydrogenases from *Desulfovibrio* spp., which can be purified aerobically and catalyse mainly H_2 oxidation;

III. Soluble, monomeric Fe-hydrogenases, found in chloroplasts of green alga *Scenedesmus obliquus*, which catalyse H_2 evolution. The [Fe_2S_2] ferredoxin functions as natural electron donor linking the enzyme to the photosynthetic electron transport chain.

Ni-Fe and Fe-only hydrogenases have some common features in their structures: each enzyme has an active site and a few Fe-S clusters. The active site is also a metallocluster, and each metal is coordinated by carbon monoxide (CO) and cyanide (CN^-) ligands.

Nath and Das (2004) have summarized the processes related to biological H_2 production and have pointed out the advantages, as well as the disadvantages of such processes (Table 1)

Process	Type of microorganism	Advantages	Disadvantages
Direct biophotolysis	Green algae	Can produce H_2 directly from water and sunlight	Requires light
		Solar conversion energy increased by tenfolds as compared to trees, crops	O_2 can be dangerous for the system
Indirect biophotolysis	Cyanobacteria	Can produce H_2 directly from water	Uptake hydrogenase enzymes are to be removed to stop H_2 dissociate
		Has the ability to fix N_2 from atmosphere	O_2 present in gas mixture, inhibits nitrogenases
Photo-fermentation	Photosynthetic bacteria	A wide spectral light energy can be used by these bacteria	Light conversion efficiency is very low – only 1-5%
		Can use different waste materials like distillery effluents, etc. and can produce H_2 all day without light	O_2 inhibits some hydrogenases
Dark fermentation	Fermentative bacteria	A variety of carbon sources can be used as substrates	Relatively lower achievable yields of H_2
		It can produce H_2 all day without light	As yields increase H_2 fermentation becomes thermodynamically unfavorable
		It produces valuable metabolites like butyric, lactic, propionic, succinic and acetic acids as by-products	Product gas mixture contains CO_2
			O_2 is an inhibitor

Table 1. Comparison of important biological hydrogen production processes (modified after Nath and Das 2004).

The hydrogen-producing microbes in a dark fermentation process can be classified into two categories: facultative anaerobes (enteric bacteria, e.g. *Escherichia coli*, *Enterobacter*, and *Citrobacter*) and strict anaerobes (clostridia, methylotrophic mathanogens, and rumen bacteria). Enteric bacteria are rod-shaped, gram-negative facultative anaerobes, less sensitive to oxygen and are able to recover following air exposure (Nath and Das 2004) the presence of oxygen, however, causes degradation of formate – a major precursor for H_2 production, without H_2 formation. Clostridia are generally obligate anaerobes and are rod shaped with round or pointed ends in some cases. Rod shape can be either straight or slightly curved with 0.5-2 µm in diameter and up to 30 µm in length. Clostridia form endospores – a survival structure, developed when the environmental conditions become unfavourable (high temperature, desiccation, carbon or nitrogen deficiency, chemical toxicity). When favourable conditions return, the spores germinate and become vegetative cells.

The H_2 production pathway of enteric bacteria during dark fermentation is presented in Fig. 3 (A). Carbohydrate-rich substrate is converted to pyruvate through glycolysis and acetyl coenzyme A is generated by pyruvate formate lyase. Formate is then converted to H_2 and CO_2 by formate hydrogen lyase. An acidic condition causes induction of lactate dehydrogenate, diverting some of the reducing power in pyruvate to lactate. This results in lower H_2 yields. The enteric bacteria produce other fermentation products like lactate, ethanol, acetate, formate, CO_2, succinate and butanediol. The maximum yield is 2 mol H_2/mol glucose.

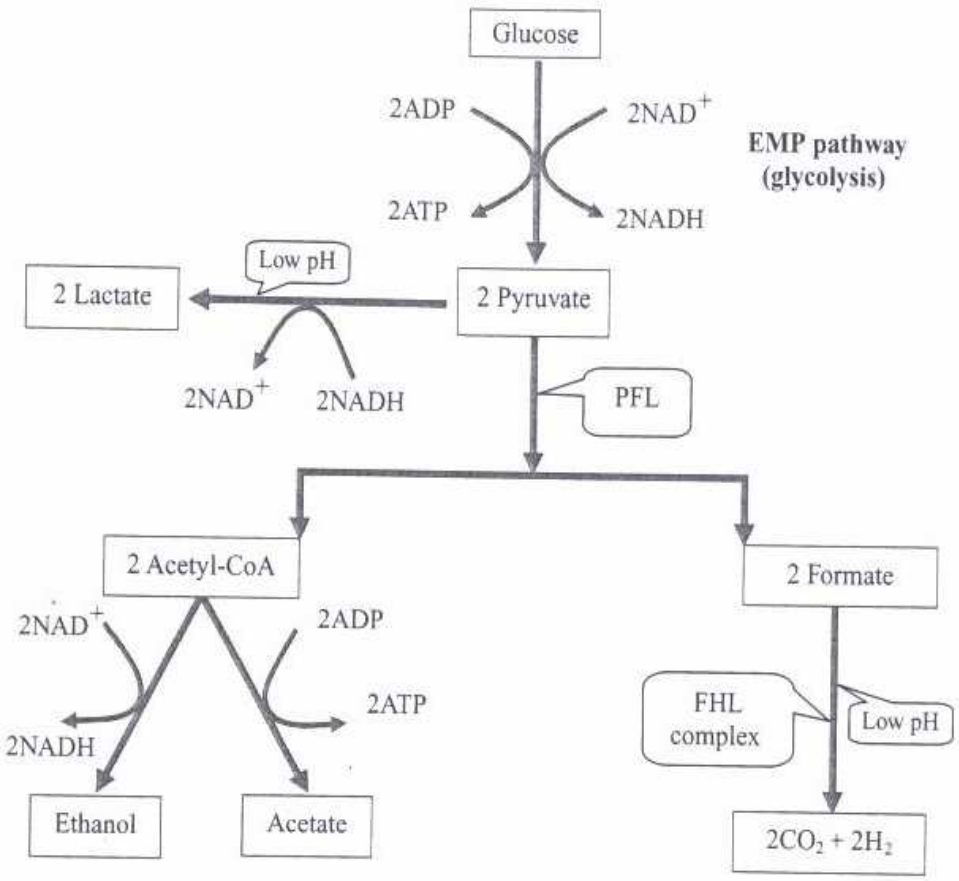


Figure 3 (A). Hydrogen production pathway of enteric bacteria during dark fermentation (adapted from Hallenbeck 2004).

The genus Clostridium has been widely studied for H_2 production. Clostridia species are capable of using different organic substrates such as proteins (Thabet et al. 2004), starch (Yokoi et al. 2001, cellulose (Levin et al. 2006), animal manure (Zhu et al. 2007) and sewage sludge (Massanet et al. 2008). Some clostridia are both proteolytic and saccharolytic and some are neither. The pathway of H_2 production using glucose as a model substrate is shown in Fig. 3 (B). Glucose is metabolized to pyruvate through glycolysis. Pyruvate is broken down into acetyl-CoA with the formation of ferredoxin (red) using pyruvate ferredoxin oxidoreductase. The reduction of a proton by ferredoxin (red) produces hydrogen through hydrogenase activity. There are a variety of end products (ethanol, acetate, acetone, butyrate, butanol, propionate, propanol) that can be generated by acetyl-CoA depending on the species and environmental conditions. H_2 production occurs mainly during the acid (acetate and butyrate) production phase. The fermentation pathway is highly dependent on pH. A maximum of 2 mol H_2/glucose can be produced in butyrate-type fermentation, whereas up to 4 mol H_2/glucose can be obtained by acetate-type fermentation.

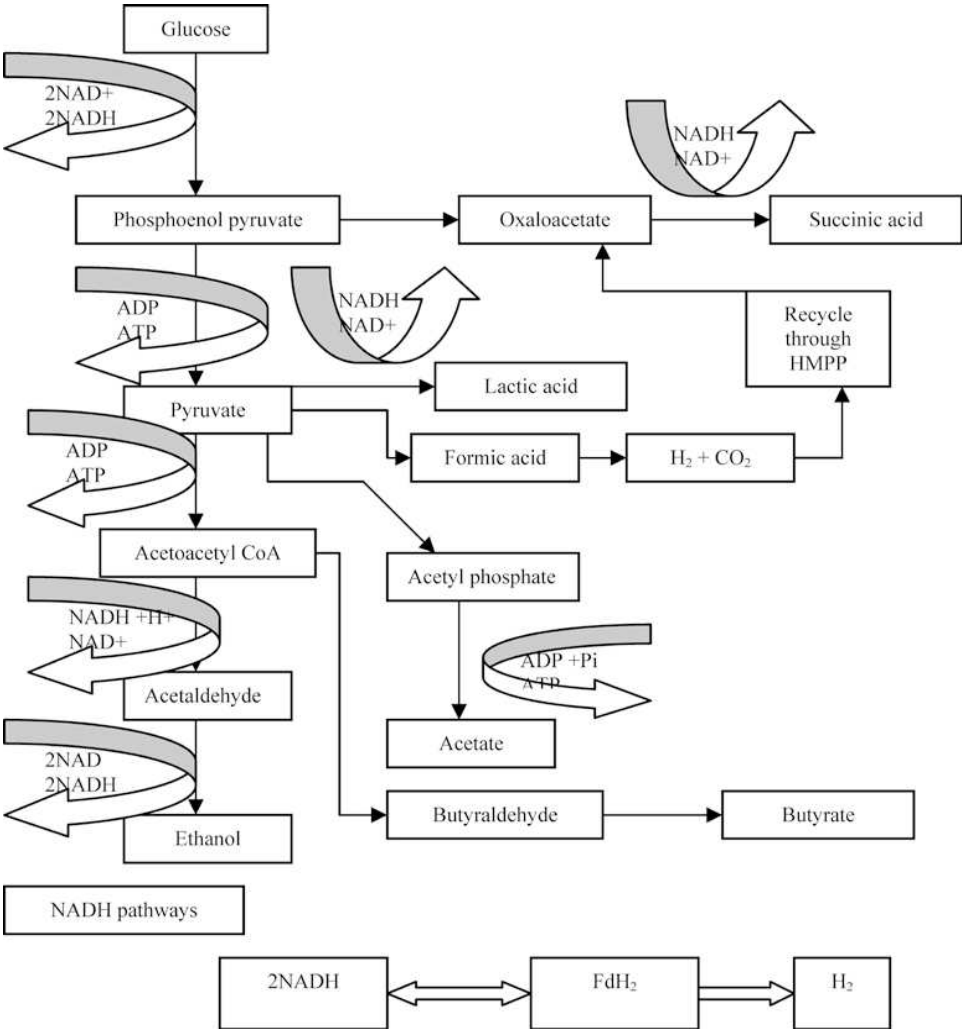


Figure 3 (B). Hydrogen production pathway of clostridia during dark fermentation (modified after Nath and Das 2004).

1.2. Hydrogen production from biomass

1.2.1. Biomass composition and methods for H_2 production from biomass

Biomass, as a product of photosynthesis, is a versatile non-petroleum renewable source that can be utilized for sustainable production of hydrogen (Table 2).

Source	Maximum output	Comment
Regenerative source		
Biomass	9×10^{12} W	For total world land coverage.
Wind power	6×10^{12} W	For total world land coverage.
Geothermal source	Perhaps 10^{9} W	Restricted to specific areas (for example, mid-ocean ridges)
Nuclear power	10^{5} W or more	No more than 1 K rise in environmental temperature. Problems of waste disposal and safety.
Fossil fuel	10^{9} W maximum allowable	Pollution abatement is essential for small and large-scale application.

Table 2. Some power sources and possible practical expectations (Nath and Das 2003).

Major resources of biomass include agricultural crops (Table 3) and their waste byproducts, lignocellulosic products such as wood and wood waste, waste from food processing, aquatic plants and algae, and effluents produced in the human habitat.

Cereals (1000 tonnes)						
	Wheat	Maize	Sorghum	Rice	Oats	Rye
World	629 873	724 515	57 924	608 368	25 828	17 650

Table 3. World production of agricultural commodities (2004). (Source: FAO, Production Yearbook Vol. 1/2, 2005/2006).

Although the debate “Food vs Fuel” criticizes the governmental subsidies for biofuels as they have raised the prices of grains dramatically and have led to deforestation in the developing countries, a new generation of "cellulosic" fuels (made from grasses, crop residue or wood shavings) might deliver benefits. Moderately-dried wastes such as wood residue, wood scrap and urban garbage can be burnt directly as fuel. Energy from water-rich biomass such as sewage sludge, agricultural and livestock effluents as well as animal excreta is

recovered mainly by microbial fermentation. Wood biomass is composed of cellulose, hemicellulose, lignin, ash and soluble substances called extractives. Cellulose and hemicellulose generally represent from two-thirds to three-quarters of the dry weight of most biomass materials. Cellulose is a polymer of glucose and is difficult to break down into glucose because of its crystalline structure. Hemicellulose is composed of several different sugars including the six-carbon sugars glucose, galactose, and mannose, and the five-carbon sugars arabinose and xylose. Hemicellulose is relatively easily broken down into its individual sugars. Lignin is a complex material that acts as glue to hold the cellulose and hemicellulose together. Extractives can have an economic value depending on their characteristics and cost of recovery. Figure 4 shows the composition of maize (called corn in US) and several other biomass materials. Wheat straw contains about thirty seven percent glucose and another twenty one percent xylose. The total sugars are comparable to those from maize.

Figure 4. Composition of wheat straw and several other biomass materials (Saha et al. 2008).

Hydrolysis is the step that breaks down the cellulose and hemicellulose polymers into their basic sugars. The major technologies proposed for hydrolysis include dilute acid hydrolysis, strong acid hydrolysis, alkaline hydrolysis and enzymatic hydrolysis. Dilute acid hydrolysis is the most advanced technology, while enzymatic hydrolysis is viewed as an environmentally – friendly technology for producing biofuels from biomass. Enzymatic hydrolysis involves microorganisms that produce enzymes degrading cellulose into sugars. In addition, using properly selected microorganisms, many agricultural feedstocks and their residues can be exploited for hydrogen production as well (Saha et al. 2008).

Different production technologies of hydrogen from biomass are being available and comparative analysis of different processes have been made on the basis of their relative advantages and disadvantages (Das and Veziroglu 2001). Nath and Das (2003) classified hydrogen production from biomass by physicochemical or biological methods as follows:

- thermochemical gasification coupled with water gas shift;
- fast pyrolysis followed by reforming of carbohydrate fractions of bio-oil;
- miscellaneous novel gasification process;
- microbial conversion of biomass.

1.2.2. Mechanism of microbial conversion of biomass to hydrogen

Despite its relatively lower yields of hydrogen, the fermentative route is a promising method of biohydrogen production due to its higher rate of H_2 evolution in the absence of any light source as well as the versatility of the substrates used. Moreover, fermentative organisms have a relatively high growth rate.

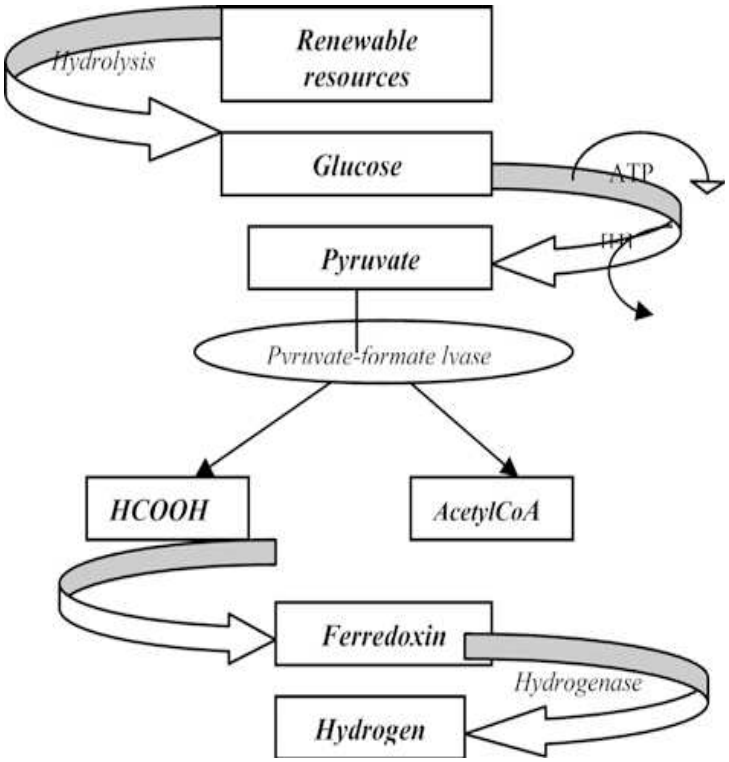


Figure 5. A schematic pathway for conversion of renewables to hydrogen via fermentation (Nath and Das 2004).

The generation of hydrogen by fermentative bacteria also accompanies the formation of organic acids as metabolic products (Fig. 5), but these anaerobes are incapable of further breaking down the acids. Acid accumulation causes a sharp drop of culture pH and subsequent inhibition of bacterial hydrogen production (Oh et al. 2002; 2003). Fermentative mesophilic bacteria like clostridia cannot sustain at pH values less than 5.0 thereby necessitating a way to reduce acid production or to carry out certain biochemical reactions that reduce the proton concentration on the outside of the cell proportional to the culture pH. In addition to volatile fatty acids, anaerobic fermentation also leads to the formation of alcohols. These reduced end-products, such as ethanol and butanol contain additional H atoms that are not liberated as gas (Levin et al. 2004). Therefore, alcohol production results in a correspondingly lower hydrogen yield.

1.2.3. Energy crops and their by-products as substrates for biofuels

Energy crops are grown specifically for their ability to generate energy. Crops such as switchgrass, hybrid poplars (cottonwoods), hybrid willows and sugarcane are being studied for their ability to serve as energy crops for fuel. One of their great advantages is that they are short rotation crops; they re-grow after each harvest, allowing multiple harvests without having to re-plant. Maize and sorghum serve a dual purpose as they can be grown for fuel, with the leftover by-products being used for other purposes, including feedstocks.

Biofuels are defined as solid, liquid and gas fuels derived from recently unviable biological material or its metabolic byproducts (organic or food waste) unlike the fossil fuels which are derived from long unviable biological material. In order to be considered a biofuel the fuel must contain over 80 percent renewable materials. It is originally derived from photosynthetic processes occurring in plants and is often being referred to as a solar energy source (Figure 6).

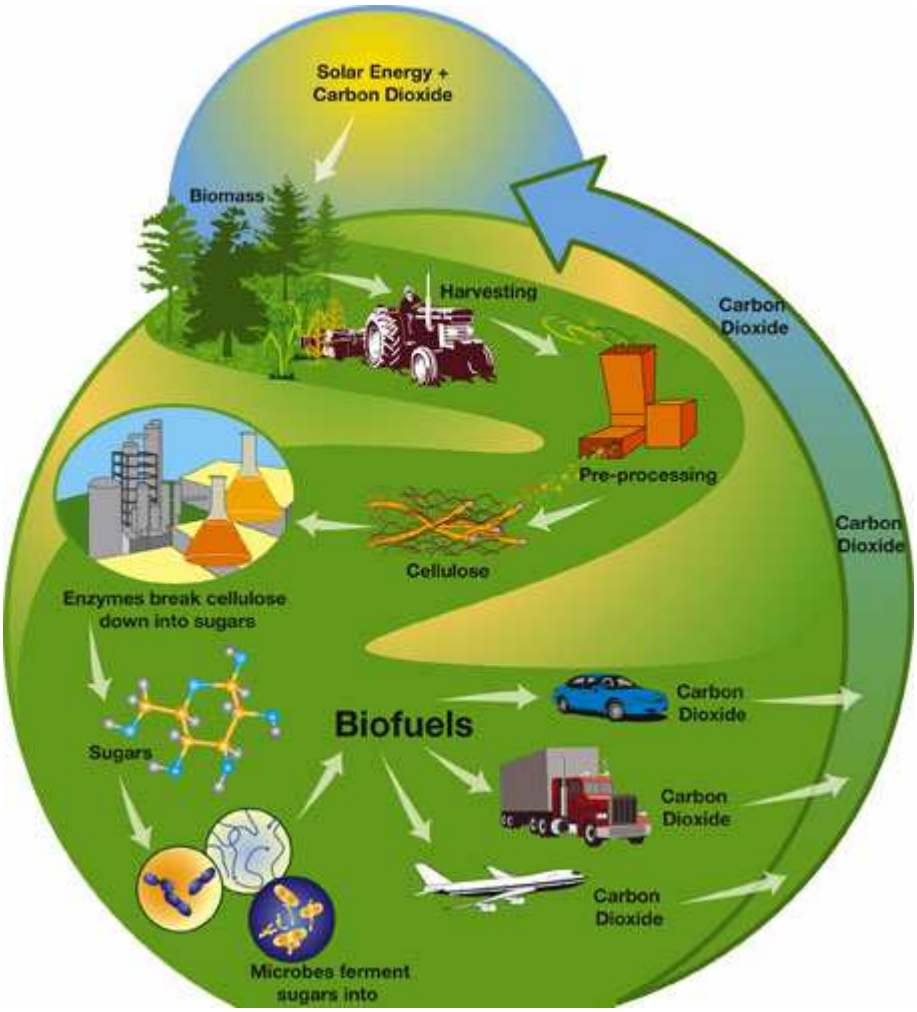


Figure 6. Biofuel conversion process (modified after the U.S. Department of Energy Joint Genome Institute and DOE Office of Science, 2008).

Biofuels have been classified into 4 main types - first, second, third and fourth generation biofuels. The most common is the first generation, including biogas, bioalcohols, biodiesel, syngas and solid biofuels like wood, sawdust, grass cuttings, domestic refuse, charcoal, agricultural waste, non-food energy crops and dried manure. Biogas refers to a gas produced by the biological breakdown of organic matter in the absence of oxygen in anaerobic digesters. One type of biogas is produced by anaerobic digestion or fermentation of biodegradable materials such as biomass, manure or sewage, municipal waste, green waste and energy crops. This type of biogas comprises primarily methane and carbon dioxide. The other principal type of biogas is wood gas which is created by gasification of wood or other biomass, comprised primarily of nitrogen, hydrogen, and carbon monoxide, with trace amounts of methane. For biogas and bioalcohols (for example bioethanol) production the

energy crops have to be pretreated since they are rich in lignocellulose. The pretreatment processes for bioethanol production involve hydrolysis, fermentation and distillation. For the biogas production the hydrolysis is followed by acido-, aceto- and methanogenesis.

Lignin is one of the drawbacks of using lignocellulosic materials in fermentation, as it makes lignocellulose resistant to chemical and biological degradation. Lignocelluloses (Figure 7) comprise not only energy plants, but also a large fraction of municipal solid waste, crop residues, animal manures and forest residues. Pretreatment by physical, chemical or biological means can enhance the bio-digestibility of the wastes for biofuel production, increasing the accessibility of the enzymes to the materials (Table 7). The best method and conditions of pretreatment depend greatly on the type of lignocelluloses and an optimisation of the process is usually implemented depending on the type of substrate used.

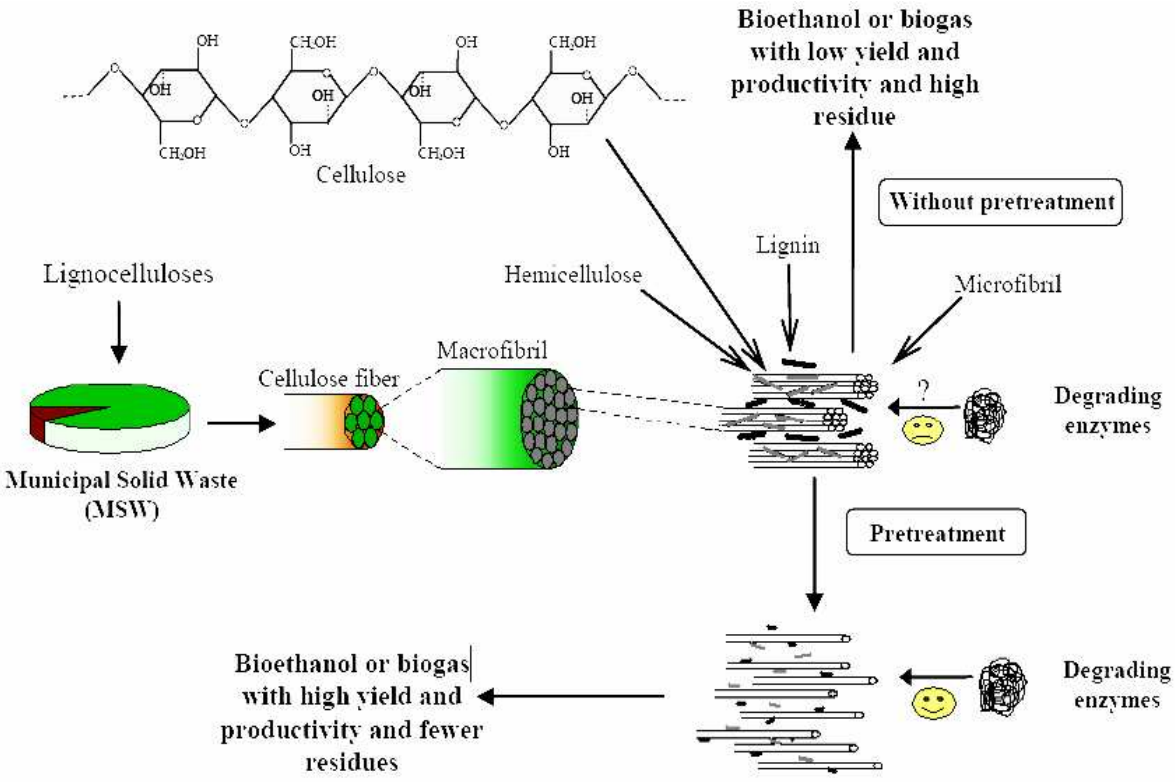


Figure 7. Effect of pretreatment on accessibility of degrading enzymes (Taherzadeh and Karimi 2008).

1.2.3.1. Biofuel production from wheat and maize residues

There are reports on biogas and bioethanol production from wheat straw. Somayaji and Khanna (1994) digested rice and wheat straw by adding cattle dung slurry, enhancing the methane production, as well as the biodegradability of organic matter. Ahring et al. (1996) produced ethanol from pretreated wheat straw by new thermophilic anaerobic isolates. Schmersahl et al. (2007) generated biogas by using cattle manure and silages of wheat straw, maize silage and grass. They concluded that the utilization of wheat straw via biogas and fuel cells can make a contribution to the power supply of 0.4 kW/ha, whereas the exclusive use of maize for biogas production might yield a constant power of up to 1.8 kW/ha.

Only in the past few years some reports on fermentative production of hydrogen from wheat and maize residues appeared in the literature. Hussy et al. (2003) produced H_2 from wheat starch co-product by using mixed microflora via a dark fermentation. Fan et al. (2006) showed an efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Pan et al. (2008) examined the degradation of wheat brans by mixed anaerobic culture. Argun et al. (2008) demonstrated biohydrogen evolution from wheat powder by anaerobic sludge. The composition of the wheat brans and the solubilised hemicellulose of the wheat have been well studied (Fang et al. 1999; Martinez-Perez 2007; Pan et al. 2008).

The distinctive features of biogas and biohydrogen formation are well described by Kovacs et al. 2004. It has been shown that by manipulation of process parameters like pH and temperature of the anaerobic fermentation biogas or biohydrogen generation can predominate (Kovacs et al. 2004; Ren et al. 2007).

Apart from being used as food and for heating, maize is currently one of the two major biofuel crops for bioethanol production (Farrel et al. 2006; Hill et al. 2006, Hammerschlag 2006; Torney et al. 2007). It is the most dominating crop for biogas production. Maize is considered to have one of the highest yield potential of field crops grown in Central Europe. The production of hydrogen from maize and their derivatives is still being at an early stage. There are few studies in that field, reporting for H_2 production using mixed microflora with H_2 yield of 62.4 mL/g dry matter fodder maize without pre-treatment (Martinez-Perez et al.

2007; Kyazze et al. 2008). Research on wheat and maize conversion to H_2 using pure thermophilic or extreme thermophilic anaerobic cultures has not been done so far.

1.2.3.2. Biofuel production from sweet sorghum and its derivatives bagasse and sweet sorghum juice

The genus *Sorghum* is characterized by a vastly diverse germ plasm in terms of phenotypic and morphological traits. Many of these have been exploited to give genotypes suitable for grain and forage production as well as alternative uses, such as energy, pulp for paper, food products, high grade chemicals and building products. It is a C4 crop, with a fibrous root system that branches profusely. Under favorable conditions, the above ground nodes may produce strong adventitious roots that may help anchor the plant and reduce lodging The roots can be extended to a distance of up to 1 m and a depth of 1.8 m. Sweet sorghum plants attain a height of up to 4 m. It is well adapted to the warm southern regions of Europe and moderately well adapted to several central European regions with mild climates. It is a cold-sensitive plant, so its adaptation in northern, cooler climates is poor. Historically, syrup production was the main use of sweet sorghum, but nowadays this crop is being used as animal fodder and is gaining attention as a potential alternative feedstock for energy and industry, because of its high yield in biomass and, particularly, fermentable sugars.

Much of the work related to non-food agricultural production of sorghum has been conducted on sweet sorghum because of the increased interest in sugar crops as potential renewable resources that can be converted into ethanol. It has a shorter growing season than sugarcane, and is therefore suitable to be grown in geographical areas with a temperate climate. It has a rapid rate of growth. In several studies, sweet types have been evaluated for fermentable sugar production and theoretical ethanol yields (Smith et al. 1987; Belletti et al. 1991), for the relationships between agronomic practices and yield (Broadhead and Freeman 1980), for optimal growth parameters (Shih et al. 1981; Tarantino et al. 1992), for the pattern of soluble carbohydrates accumulation (Ferraris and Charles-Edwards 1986; Petrini et al. 1993), and for relevant physiological aspects of this metabolic process (Vietor and Miller 1990). Sweet sorghum can be converted into energy carriers through either one of two pathways: biochemical and thermo-chemical. Through biochemical processes the crop sugars

can be converted to biofuels (ethanol, hydrogen). Thermo-chemical processes such as combustion and gasification can be used for the conversion of the sweet sorghum bagasse (the residual cake from crop pressing) to heat and electricity (Claassen et al. 2004). Unlike sweet genotypes, nonsweet, and especially those characterized by stalk storage organ with high fiber content, have had little attention so far.

Mamma et al. (1996) saccharified and fermented sweet sorghum carbohydrates to ethanol by a mixed culture of *Fusarium oxysporum* and *Saccharomyces cerevisiae* in a bioreactor. *Fusarium oxysporum* was grown aerobically for the production of the enzymes necessary for the saccharification of sorghum cellulose and hemicellulose.

Although sweet sorghum has been thoroughly investigated as an energy crop for bioethanol production, it was just recently tested as a potential source for biogas and hydrogen production. The technologies for production of biogas from sweet sorghum is still on R&D level but some results have been recently published by Antonopolulou et al. (2007; 2008). Their study showed that sweet sorghum extract could be used for hydrogen and methane production in a two-stage process. They proved that the effluent from the hydrogenogenic reactor was an ideal substrate for methane production with approximately 107 L CH_4/kg sweet sorghum, 78 L of which come from the solid residues. The authors demonstrated that biohydrogen production can be very efficiently coupled with a subsequent step of methane production and that sweet sorghum could be an ideal substrate for a combined gaseous biofuels production.

Ntaikou et al. 2008 used *Ruminococcus albus* chemostat cultures at 37 °C to produce H_2 from sorghum stalks, sorghum water extract, containing the free sugars, and sorghum residues after the extraction process as substrates The hydrogen yield from sorghum water extract was the same as the yield obtained from glucose batch experiments (approximately 2.5 mol H_2/mol glucose). The hydrogen productivity of sorghum extract plus that of sorghum residues equalled the hydrogen productivity obtained from the sorghum stalks, suggesting that the process could be designed as a single-step process, avoiding the separate fermentation of soluble and insoluble carbohydrates as well as the extraction process, without any compromise in the hydrogen productivity. Hydrogen productivity was estimated to be approximately 60 L of hydrogen per kg of wet sorghum biomass, proving that *R. albus* is suitable for efficient hydrogen production from sweet sorghum biomass.

Bagasse is the fibrous biomass (cake) remaining after sorghum or sugarcane stalks are pressed to extract their juice. In a sugarcane mill, freshly harvested sugarcane is shredded, mixed with water, and crushed between heavy rollers to extract the juice, which is high in sucrose (10-15%). The residue is used as a tree-free alternative for making paper. The process requires no bleaching, it is biodegradable, easy to recycle and overall has less polluting affect on the environment.

Bagasse is widely used for ethanol production from the sugar in sugarcane in Brazil. The cellulose rich bagasse is currently being tested for production of cellulosic ethanol by using yeasts and genetically modified bacteria (Martin et al. 2002; Dien et al. 2003; Boopathy 2005; Dawson and Boopathy 2007; 2008). The major limitations to efficient ethanol production from bagasse come from the close physical and chemical associations between lignin and plant cell wall polysaccharides, together with cellullose crystallinity. To convert the biomass into ethanol, the cellulose must be readily available for cellulase enzymes, which can be made possible by removing the lignin. Most reports on lignocellulosic ethanol involve acid hydrolysis followed by expensive enzymatic saccharification (Martin et al. 2002). The enzymatic step has been omitted by using recombinant and mutant bacteria (Dien et al. 2003; Kim et al. 2007).

Bagasse has been extensively used in the past years for biogas production (Dellepiane et al. 2003; Chinnaraj and Rao 2006). Dellepiane et al. (2003) discuss the economic advantages and disadvantages of using bagasse for bioenergy in Latin America, a big producer of sugarcane. Chinnaraj and Rao (2006) are regarding bagasse as a possibility to reduce the methane and CO_2 emissions in the atmosphere by using it in upflow anaerobic sludge blanket digesters to replace the conventional anaerobically treated bagasse in lagoons.

Most of the hydrogen produced so far has been done by steam methane reforming of natural gas for fuel cell vehicles. At low natural gas prices it is the least expensive way to produce hydrogen but it relies on a non-renewable fossil fuel. Bowen et al. (2003) made a techno-economic analysis of hydrogen production by gasification of three biomass feedstocks: bagasse, switchgrass and nutshell mix. They concluded that H_2 can be produced economically from biomass gasification, with prices from \$6.50 to \$10.00 per 1 GJ compared to \$5.50 – \$7.50 per 1GJ H_2 from methane reforming. Chaudhari et al. (2003) produced

hydrogen and/or syngas from bagasse char and their results indicated that there was a strong potential for H_2 production using that method at temperatures of 700-800 °C and steam flow rates of 1.25 to 10 g/h/g of bagasse char.

There are only few reports on biohydrogen production from sweet sorghum bagasse and sweet sorghum juice. Bagasse has been pretreated with an acidic and alkaline catalyst for increasing the biomass fermentability. The best results were obtained after the alkaline pretreatment where 37.1 g glucose, corresponding to 60% cellulose conversion was obtained from 100 g of original bagasse (Claassen et al. 2004). Sweet sorghum juice was also used for biohydrogen production. Stalks were cut to 5-10 cm pieces and then milled. Three different procedures were tested in order to produce the juice which was used as substrate for hydrogen production: 1) pressing; 2) pretreatment and pressing; 3) water extraction and pressing the residue. For the pretreatment a central composite design was applied with four design factors, namely the temperature and the duration of the pretreatment, the pressure and the amount of water added to the sample before pressing. The second pretreatment gave the best results. Sweet sorghum juice supported the growth and H_2 production by the extreme thermophile *Caldicellulosiruptor saccharolyticus* for about 60 hours with an average production rate of 10 mmol H_2/L.h during the first 16 hours and a maximal production rate of 21 mmol H_2/L.h 10 hours after the start of fermentation. The authors concluded that the production of biohydrogen had showed great promise to convert biomass such as sweet sorghum and its byproducts to a pure hydrogen stream. Future optimisations of the micro-organisms and system efficiency as well as research and development of process parameters such as bioreactors' design are needed.

1.3. The extreme thermophile Caldicellulosiruptor saccharolyticus

1.3.1. Characteristics of the extreme thermophiles (extremophiles)

Heat-loving microbes, or thermophiles reproduce readily in temperatures greater than 45 °C, and some of them, referred to as hyperthermophiles favour temperatures above 75-80

°C, up to 100 °C or even higher. Thermophiles that grow at temperatures up to 60 °C have been known for a long time, but true extremophiles were first discovered only 40 years ago in 1968 by Thomas Brock at the University of Wisconsin, Madison, USA. He isolated microorganisms growing at 80°C and higher in hot springs at Yellowstone National Park. Among them was the bacterium *Thermus aquaticus* that later made possible the widespread use of a revolutionary technology – the polymerase chain reaction (PCR) (Madigan and Marrs, 1997). Hyperthermophiles have been isolated almost exclusively from environments with *in situ* temperatures between about 75-80 and 115°C. Natural biotopes of hyperthermophiles on land are water containing volcanic areas, hot springs, with low salinity and a wide range of pH values (around pH 0.5–8.5). Marine biotopes are shallow submarine hydrothermal systems, abyssal hot vents ('Black Smokers'), and active seamounts. These environments contain high concentrations of salt (around 3%) and the pHs are slightly acidic to slightly alkaline (pH 5–8.5). In addition, smoldering coal refuse piles and geothermal power plants are man-made biotopes for hyperthermophiles (Fuchs et al., 1995). Communities of hyperthermophiles have also been discovered within oil-bearing deep geothermally heated soils. Due to the low solubility of oxygen at high temperatures and the presence of reducing gasses, biotopes of hyperthermophiles are mainly anoxic. Although unable to grow, hyperthermophiles may survive for long times at ambient temperature. This ability may be essential for dissemination through the cold atmosphere and hydrosphere. Hyperthermophiles are well adapted to their biotopes. Within their habitats, they form complex ecosystems consisting of a variety of primary producers and decomposers of organic matter. Primary producers are chemolithoautotrophs using inorganic electron donors and acceptors in their energy-yielding reactions. The 16S rRNA-based universal phylogenetic tree shows a tripartite division of the living world consisting of the domains *Bacteria*, *Archaea* and *Eucarya* (Woese et al. 1990; Fig. 8). The root is derived from phylogenetic trees of duplicated genes of ATPase subunits and elongation factors Tu and G (Iwabe et al. 1989). Short phylogenetic branches indicate a rather slow distance of evolution. Deep branching points are evidence for early separation of two groups. Surprisingly, all the deepest and shortest lineages within the universal phylogenetic tree are represented by hyperthermophiles, including *Aquifex* and *Thermotoga* within the *Bacteria*; *Pyrodictium*, *Pyrolobus*, *Pyrobaculum*, *Desulfurococcus*, *Sulfolobus*, *Methanopyrus*, *Thermococcus*, *Methanothermus*, *Archaeoglobus* within the Archaea (Fig. 8, bold lines). Based on these observations, hyperthermophiles appear to be the most primitive organisms still existing and the first common ancestor may have been a hyperthermophile (Stetter 1994).

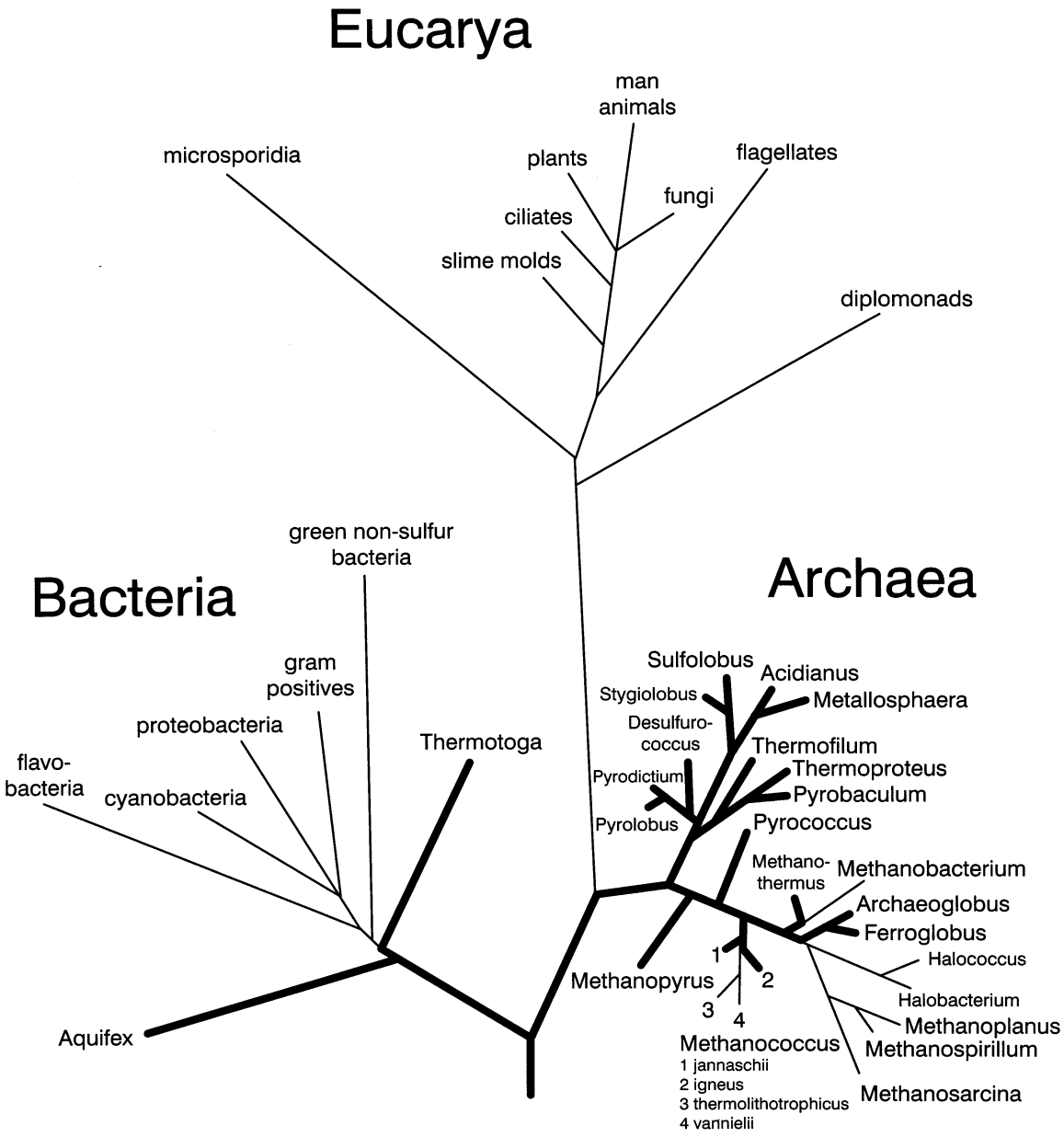


Figure 8. Hyperthermophiles within the phylogenetic tree; schematically redrawn and modified from Woese et al. 1990.

The biochemical basis of heat stability of hyperthermophiles is still under investigation. Membrane lipids of *Thermotoga maritima* are based on n-fatty acids, diabolic acids (15,16-dimethyltriacontanedioic acid) and a novel glycerol ether lipid 15,16-dimethyl-30-glyceryloxy triacontanedioic acid (De Rosa et al. 1989). The presence of an ether lipid with an unprecedented structure may significantly increase stability of membranes against hydrolysis at high growth temperatures.

In view of an enormous, so far uncultivated variety of hyperthermophiles (Barns et al. 1994), a further strategy to obtain novel enzymes could be based on DNA extraction directly from the environment, followed by gene expression, robot screening, and cloning in production strains of (mesophilic) microorganisms (Robertson et al. 1996). By comparison of sequences of homologous enzymes, properties can be assigned to specific gene segments which may be important for future enzyme design.

The hyperthermophiles' enzymes are gaining new fields of applications. A major commercial application of a thermostable enzyme so far is the polymerase- chain-reaction (PCR) employing the DNA polymerase of *Thermus aquaticus.* In the future the whole variety of hyperthermophilic isolates may serve as a source of enzymes with very different properties. For example, the DNA polymerase of *P. furiosus* (Pfu) as well as that of *T. maritima* (UITma™) with their higher fidelity due to proofreading, are already commercially available now. Thermophilic hydrolases such as proteases, lipases, amylases, and xylanases are of interest of several branches of industry for detergents, starch processing, paper mills, and dairy products. Novel metabolites can give rise to new pharmaceuticals. For example, *Thermococcus* forms organic sulphur compounds related to lenthionin, some of which are pharmaceutically active (Ritzau et al. 1993). Oil-degrading hyperthermophiles form surfactants, suitable for oil recovery. In addition, hyperthermophiles are directly applied in technical processes like bioleaching of sulphide ores and in coal desulphurization. In biohydrometallurgy representatives of *Sulfolobus, Acidianus* and *Metallosphaera* are applied in leaching processes. This includes the treatment of refractory gold ores (by removing the pyrite or arsenopyrite), the leaching of low grade ores (e.g. copper, zinc ores) or the removal of pyrite from coal. The high turnovers, compared to mesophilic leaching organisms (Huber and Stetter 1991) recommend hyperthermophiles even for reactor leaching of ore concentrates in continuous processes. High temperatures arise due to self-heating during the biological leaching process. Within heaps temperatures up to 80°C have been measured and in reactor leaching the costs for cooling can be saved by using the archaeal hyperthermophilic leaching organisms.

1.3.2. Properties of *Caldicellulosiruptor saccharolyticus* (*C. saccharolyticus)*

H_2 production by strict anaerobic, thermophilic and extreme thermophilic bacteria has not been fully elucidated yet. The number of reports on these topics continues to increase (Adams 1990; Li and Borchardt 1995; Kengen et al. 1996; Ueno et al. 1996 and Rainey et al. 1994). *Caldicellulosiruptor saccharolyticus* has been first discovered by Rainey et al. (1994) and described as an obligately anaerobic, extreme thermophilic rod-shaped bacterium, isolated from thermal springs at 70-75 °C. It has attracted considerable attention by virtue of its ability to degrade various polysaccharide substrates (e.g. amorphous cellulose and starch), disaccharides (e.g. cellobiose and sucrose) and monosaccharides (e.g. glucose, fructose and xylose) (Rainey et al. 1994; Van Niel et al. 2002) at temperatures above 70 °C. The multifunctional, multidomain organization of the majority of *C. saccharolyticus* β-glycanases (e.g. β-glucosidase and endo-1,4-β-mannosidase) (Bergquist et al. 2000), xylanases and cellulases (Bergquist et al. 1999; Gibbs et al. 2000 and Sunna et al. 2000) makes this strain a unique candidate for biotechnological exploitation. In consequence of its ability to convert various sugars to H_2 *C. saccharolyticus* has been selected for the fermentative production of H_2 from sucrose (Van Niel et al. 2003), from industrial waste paper sludge (Kadar et al. 2004), and from sources such as domestic organic waste, agro-industrial residues and energy crops (Claassen et al. 1999). Its production rates are superior to those of the mesophiles (Adams 1990). In anaerobic digestion H_2-producing bacteria usually function in syntrophy with hydrogenotrophic methanogens, which consume H_2 (Claassen 2005). In this respect, *C. saccharolyticus* can also be employed in biogas production technologies on the basis of its ability to utilize pentose and hexose sugars (Bagi et al. 2007).

In contrast to mesophilic fermentative anaerobes, *C. saccharolyticus* produces almost no reduced end products, such as lactate or ethanol, and the amount of hydrogen approaches 4 mol H_2/mol glucose. The complete genome sequence of *C. saccharolyticus* provides new insights into the exceptional capacity of that bacterium to degrade a variety of plant polysaccharides and further reveals its plasticity with many transposases, sugar hydrolases and transferases. Metabolic pathways for the degradation of residual components of cellulose, hemicellulose, starch and pectin could be assigned (Fig. 9 (A)). Reducing equivalents are produced as NADH or reduced ferredoxin, which are used to produce H_2 by a soluble NADH-

dependent Fe-only hydrogenase and a membrane-bound ferredoxin-dependent [NiFe] hydrogenase (Fig. 9 (B)).

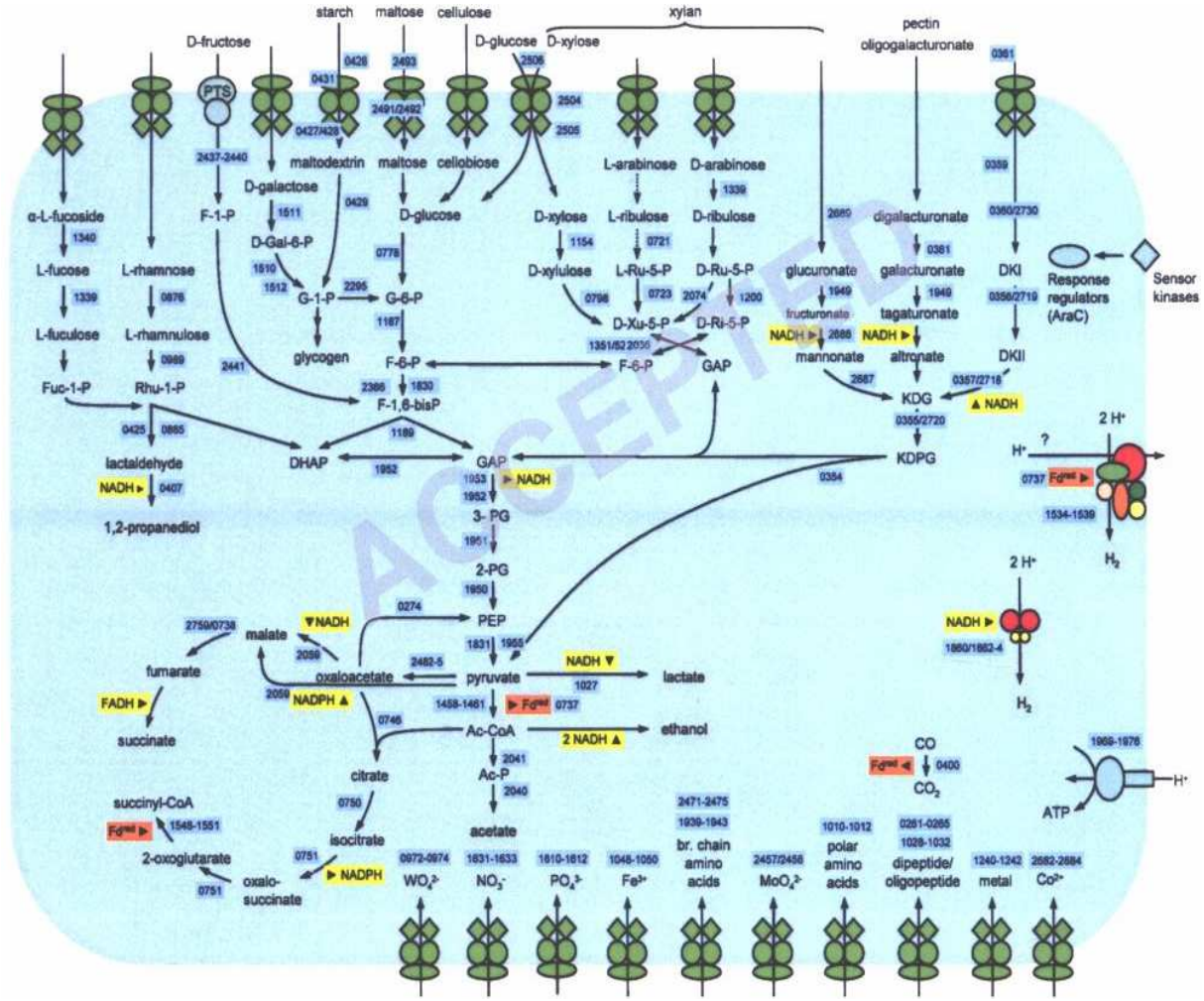


Figure 9 (A). Central catabolic pathways of *C. saccharolyticus* (van de Werken et al. 2008)

The ability to produce H_2 directly from NADH is not known for mesophilic anaerobes and may be responsible for the relatively high H_2 production rates by *C. saccharolyticus*. In mesophiles, reducing equivalents from NADH first have to be transfered to ferredoxin, which requires input of energy, either by a sodium gradient or by coupling to an exergenic reaction (Li et al. 2007). In extreme thermophiles, such as *C. saccharolyticus*, this is apparently not necessary (van de Werken et al. 2008).

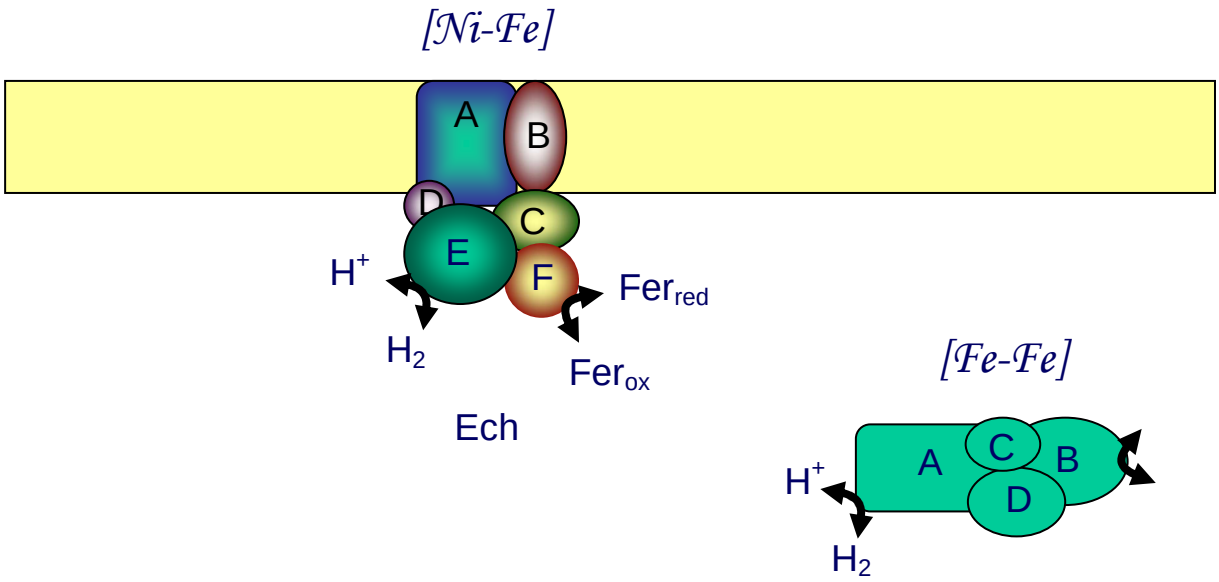


Figure 9 (B). Hydrogenases model for *C. saccharolyticus* (Soboh et al. 2004).

The sequence identity of *C. saccharolyticus*[NiFe] hydrogenase (Ech subunits) has been compared to other bacteria and it was concluded that it is the closest to *Thermoanaerobacter tengcongensis* (Table 4).

	Sequence identity to *Thermoanaerobacter tengcongensis* (%)	Sequence identity to *Methanosarcina barkeri* (%)	Sequence identity to *Desulfovibrio gigas* (%)
EchA	44	40	42
EchB	46	49	50
EchC	69	68	67
EchD	50	48	49
EchE	68	57	60
EchF	49	51	43

Table 4. Sequence identity to known Ech subunits in *C. saccharolyticus* (Hedderick 2004).

The absence of catabolite repression by glucose is an important characteristic for biohydrogen producers since it allows them to process an array of biomass-derived substrates simultaneously. Glucose did not repress the use of xylose in *C. saccharolyticus*. The EM pathway was not affected by the hexose or pentose substrate, in contrast to the transcriptome analysis of *E. coli* (van de Werken 2008).

1.4. Rationale of whole cell immobilization and its application in biotechnology

1.4.1. Methods for immobilization of whole cells and their application in biotechnology

Immobilized cell technologies have widely developed since the early 1960s, and thousands of such documents are currently available. Immobilized cell (IC) systems can be separated into artificial and naturally occurring ones. In the first category, microbial (or eukaryotic) cells are artificially entrapped in or attached to various matrices/supports where they keep a viable state, depending on the degree of harm of the immobilization procedure. Polysaccharide gel matrices, more particularly Ca-alginate hydrogels (Gerbsch and Buchholz 1995), are by far the most frequently used materials for cell entrapment. The main benefit derived from the use of whole cells instead of enzymes was to avoid enzyme extraction and purification steps and their consequences on enzyme activity, stability and cost. As viable ICs are able to multiply during substrate catabolism while remaining confined within the immobilization structures (polysaccharide gel matrix of artificially gel-entrapped cells) high cell densities may be expected in IC cultures, leading to high volumetric reaction rates. The ability to grow in an immobilized state makes the regeneration of IC cultures possible after having them incubated under hostile conditions such as low-nutrient medium or presence of toxic compounds.

Cell attachment to an organic or inorganic support may be obtained by creating chemical (covalent) bonds between cells and the support using cross-linking agents such as glutaraldehyde or carbodiimide. The spontaneous adsorption of microbial cells to different types of carrier gives natural immobilized cell systems in which cells are attached to their

support by weak (non-covalent), generally non-specific interactions such as electrostatic interactions. In suitable environmental conditions, this initial adsorption step may be followed by colonization of the support, leading to the formation of a biofilm in which microorganisms are entrapped within a matrix of extracellular polymers they themselves secreted. Owing to the presence of this polymer paste, biofilms are more firmly attached to their substratum than merely adsorbed cells. Surface colonization to form biofilms is a universal bacterial strategy for survival, and undesirable biofilms may occur on inert or living supports in natural or biological environments as well as in industrial installations. The last 10 years have known a burst in the number of published investigations on these natural immobilized cell systems (Fig. 9B).

As detailed in Table 5, a large part of published data on artificial or natural IC systems concerns their operation in bioreactors where they perform biosyntheses or bioconversions leading to a variety of compounds, ranging from primary metabolites to high-value biomolecules. IC cultures have also been widely applied to the treatment of domestic or industrial wastewaters containing different types of pollutants such as nitrate/nitrite ions, heavy metals or organic compounds recalcitrant to biodegradation. Together with brewing and wine-making processes, biosensors for environmental monitoring, food quality analysis and fermentation process control complete the main application fields of ICs. Faced with these dominant and prolific developments, research on the physiological behaviour of microbial cells in the immobilized state remains paradoxically limited.

Biosyntheses, bioconversions	
Enzymes	α-Amylases, cellulase and other cellulolytic enzymes, chitinolytic enzymes, cyclodextrin glucosyltransferase, L-glutaminase, inulase, lipases, penicillin V acylase, peroxidases, polymethylgalacturonase, alkaline and acid proteases, pullulanases, ribonuclease, xylanase
Antibiotics	Ampicillin, candicidin, cephalosporin C, clavulanic acid, cyclosporin A, daunorubicin, divercin, kasugamycin, nikkomycin, nisin Z, oxytetracyclin, patulin, penicillin G, rifamycin B
Steroids[a]	Androstenedione, hydrocortisone, prednisolone, progesterone
Amino acids	Alanine, arginine, aspartic acid, cysteine, glutamic acid, phenylalanine, serine, tryptophan
Organic acids	Acetic, citric, fumaric, gluconic, lactic, malic, propionic acids
Alcohols	Butanol, ethanol, sorbitol, xylitol
Polysaccharides	Alginate, dextran, levan, pullulan, sulfated exopolysaccharides
Varia	Pigments, vitamins, flavors and aroma
Environment	
Water treatment	Carbon removal (COD), nitrogen removal (nitrification/denitrification, assimilation), heavy metal removal (Au, Cd, Cu, Ni, Pb, Sr, Th, U, . . .), pollutant biodegradation (phenol and phenolic compounds, polycyclic aromatics, heterocycles, cyanide compounds, surfactants, hydrocarbons, oily products)
Biofertilisation	Soil inoculation with plant growth-promoting organisms (*Azospirillum brasilense*, *Bradyrhizobium japonicum*, *Glomus deserticola*, *Pseudomonas fluorescens*, *Yarowia lipolytica*)
Bioremediation	Degradation of pollutants in contaminated soils (e.g. chlorinated phenols), aquifers and marine habitats (e.g. petroleum hydrocarbons) by microbial inocula
Alternative fuels	Dihydrogen and methane productions, ethanol production, biofuel cells
Food processing	
Alcoholic beverages	Brewing, vinification, fermentation of cider and kefir; controlled in situ generation of bioflavors
Milk products	Continuous inoculation of milk (lactic starters), lactose hydrolysis in milk whey
Biosensors	
Electrochemical[b]	Acetic acid, acrylinitrile, amino acids, BOD, cyanide, cholesterol, chlorinated aliphatic compounds, ethanol, naphthalene, nitrate, phenolic compounds, phosphate, pyruvate, sugars, sulfuric acid (corrosion monitoring), uric acid, herbicides, pesticides, vitamins, toxicity assays
Optical	Herbicides, metals, genotoxicant, polyaromatics, toxicity testing

[a] Obtained by conversion of steroid parent compounds.
[b] Amperometric, potentiometric, conductometric.

Table 5. Main application fields of IC cultures (Junter and Jouenne 2004).

The use of biomass attached to or entrapped in particulate carriers ensures efficient biomass retention in the reactor during continuous processes, minimizing cell washout that occurs at high dilution rates and limiting the volumetric conversion capacity of classical, free-cell-based continuous stirred tank reactors (i.e. chemostats). Continuous IC bioreactors can

therefore be operated at high load, even when diluted feeds are used: a definite advantage in wastewater treatment. Easier downstream processing, due in particular to facilitated cell/liquid separation, represents another asset of fermentation processes using IC cultures. From the outset of IC technology, enhanced operational and storage stabilities have been presented as a key feature for practical development of viable IC systems. These stabilities involve both biological and mechanical characteristics of IC biocatalysts. In order to explain the increase in the biological stability of ICs Dervakos and Webb (1991) proposed several hypotheses based on IC's ability to grow. Biological stabilization was explained by a prolonged operational time and improved resistance to storage periods. Long-term biological activities were allowed by alternate operations of ICs between growth and non-growth conditions, adapted to non-growth-associated processes, as well as by periodic rejuvenation of the biocatalyst in nutrient-rich medium.

A major characteristic of ICs is their high resistance to environmental stresses, in particular, the exposure to toxic compounds. The biodegradation of toxic compounds, pollutants and xenobiotics also represents a preferential application field of IC systems. The high biodegradation efficiency and operational stability of IC cultures, highlighted for instance, during continuous biodegradation assays of phenol and phenolic derivatives is typically ascribed to some protecting effect of the immobilization support (Dervakos and Webb, 1991), rather than to enhanced specific degradation capacity that might involve physiological modifications in ICs. In the case of the widely investigated biodegradation of phenol, several authors have implied reversible adsorption of the pollutant on the immobilization matrix (Cassidy et al., 1997; Annadurai et al., 2000).

The potential advantages of IC systems over conventional fermentations can be summarized as follows:

- Higher reaction rates due to increased cell densities;
- Possibilities for regenerating the biocatalytic activity of IC structures;
- Ability to conduct continuous operations at high dilution rate without washout;
- Easier control of the fermentation process;
- Long-term stabilization of cell activity;
- Reusability of the biocatalyst;
- Higher specific product yields.

The technological obstacles to a large-scale industrial implementation of IC systems have also been regularly investigated, with particular emphasis on the mass transfer limitations inside immobilization matrices and the coupled transport-reaction phenomena that control the performance of IC cultures. Therefore, it appears that the initial rationale for IC development essentially concerned the engineering level, with very few - if any - queries on the physiological behaviour of microbial cultures in the immobilized state. This historical prevalence of applications over more basic investigations may explain why our present knowledge of IC physiology still remains fragmentary. A major physiological characteristic of ICs is their high resistance to environmental stresses, in particular, the exposure to toxic compounds. Some reports connect this resistance to changes in structural features affecting IC permeability, namely the composition and organization of the cell wall and the plasma membrane (Jirku 1999). The high biodegradation efficiency and operational stability of IC cultures, highlighted for instance, during continuous biodegradation assays of phenol and phenolic derivatives (Table 5), is typically ascribed to some protecting effect of the immobilization support (Dervakos and Webb, 1991), rather than to enhanced specific degradation capacity that might involve physiological modifications in ICs. A variety of bacteria at surfaces and within biofilms have been shown to display altered gene expression as compared to planktonic organisms (Whiteley et al. 2001; Schembri et al. 2003). Most proteomic analyses of biofilm cells consists of comparing the crude protein patterns of organisms cultured in the sessile (immobilized) and planktonic (suspended) modes. These studies have revealed some alterations in the bacterial protein profiles ranging from 3% to more than 50% of the detected protein spots which gives evidence of significant physiological differences between the two modes of growth.

Microorganism	Biofilm		Number of spots/gel	Number of modified spots[a]		Change (%)
	Substratum	Age		+	–	
Bacillus cereus	glass wool fibres	2 h	345	19	4	7
		18 h		26	8	10
Campylobacter jejuni	glass beads	48 h	n.g.	12	7	–
Escherichia coli	glass fibre membrane filters	7 days	600	14	3	3
E. coli	glass beads	2 h	38[b]	17	15	84
Listeria monocytogenes	glass fibre membrane filters	7 days	550	22	9	6
P. aeruginosa	glass wool fibres	18 h	844	49	48	11.5
		48 h	838	182	47	27
P. aeruginosa	clay beads	18 h	816	48	130	22
		48 h	841	62	78	17
P. aeruginosa	silicone tubing	1 day	≈ 1500	375	60	29
		6 days		765	90	57
Pseudomonas putida	silicone tubing	6 h	1000	15	30	4.5
Streptococcus mutans	epon-hydroxyapatite rods	3 days	694	57	78	19.5

[a] (+) Overproduced; (–) underproduced.
[b] Outer membrane proteins.

Table 6. Number of proteins whose amount was reported to be modified in biofilm cells as compared to planktonic organisms, (Junter and Jouenne 2004).

Proteomic analyses of artificially immobilized bacteria are much scarcer. The total protein contents of agar entrapped *E. coli* cells incubated for 2 days in a minimal nutrient medium were compared to those of suspended cells harvested during the exponential or the stationary phase of growth (Perrot et al. 2000). The results showed that gel-entrapped cultures cannot be likened to ordinary stationary-phase cell systems. The protein-based approaches to IC physiology, suggesting that many genes are differentially regulated during culture development in the immobilized state, in contrast to transcriptome analyses from which only a few genes show altered expression as a consequence of bacterial adhesion (Whiteley et al. 2001; Schembri et al. 2003). As discussed by Ghigo (2003) in a recent review, however, this modest overlap between results of proteomic and transcriptomic studies is not surprising, since the relationships between mRNA and protein contents are heavily dependent on time, cellular localization and the stability of molecules. Furthermore, the thresholds used to define

over - and down - regulations in both transcriptomic and proteomic analyses suffer from the lack of standardization, which may contribute to these discrepancies.

1.4.2. Immobilization of whole cells by adsorption

Compared to the immobilization of cells by entrapment into organic polymers, the adsorption is a process, suitable mainly for viable cells. The binding of the cells is rendered by Van der Waals forces and ionic or covalent interactions and sometimes microbial exopolymers are involved in the process as well. The metabolic status of the adsorbed cells is important. In identical environment different kind of organisms show a different adsorption behaviour (Ellwood et al. 1982). Hattori and Hattori (1987) distinguished between strongly and loosely attached *E. coli* cells while before the adsorption experiment all cells were in the same physiological state. A direct correlation between growth rate and adsorption was observed with *Pseudomonas* cells in continuous culture (Nelson et al. 1985). Very high adsorption rates have been obtained at a very low dilution rate, corresponding to low growth rate.

The selection of the adsorption matrix should be influenced by several parameters. The material has to be cheap and available in large amounts and the immobilization process must be simple and effective in regard to yield and retaining the cell activities. Cheap inorganic (fired bricks, sand particles, ceramics, metallic hydroxides and porous glass) and organic carriers (charcoal, wood shavings and organic polymers like collagen, cellulose, carrageenan, alginate, albumin, polyvinylchlorides, polypropylene, polysaccharides, ion exchange resins, epoxides and polyurethanes) are widely available. Adsorption has to be preferred, being the more simple immobilization method. That was proved by using porous glass or lava-based fixed-bed reactors in the fields of aerobic (Bohnke 1981) and anaerobic (Aivasidis 1984) waste water purification, for gas elimination in the form of trickling filters and biofilters (Ottengreaf 1987), or in environmental technologies and vinegar production.

2. Aim of the study

Hydrogen has been accepted as an attractive, clean and renewable energy carrier. Since it is unavailable in nature, inexpensive production methods are to be established. In order to replace the conventional fossil fuels, depleting at a fast speed, with environmentally friendly and economically feasible resources for hydrogen production, new substrates and new biological activities need to be found. This research has been urged by the need of recognizing such resources - cheap, widely spread or discarded as wastes, which are readily degradable by the hydrogen-producing extremophile *C. saccharolyticus*.

In addition to identifying new substrates for H_2 production, requiring adequate enzymatic activities of *C. saccharolyticus*, optimal conditions for the storage of *C. saccharolyticus* on suitable immobilization support matrices were targeted. They were aimed to preserve the hydrogen producing capacity and the survival of the cells to the best extent.

More specifically, my study had the following main goals:

1. To demonstrate that *C. saccharolyticus* has diverse fermentation pathways for utilization of energy-rich biopolymers, discarded as wastes in the environment.
2. To select widely available and cheap polysaccharidic and cellulolytic substrates, which are readily metabolized by *C. saccharolyticus*, producing hydrogen.
3. To screen for suitable, non-toxic and cheap solid immobilization support matrices to stabilize the hydrogen-producing system.
4. To establish the optimal storage conditions for *C. saccharolyticus*.
5. To improve the productivity and prolong the storability of the immobilized *C. saccharolyticus* cells.
6. To select for support matrices, which have a duel function for the cells - ones, which not only provide a solid surface for the cells, but also play a role of a substrate, which maintains the viability and preserve the physiology of stored cells over time.

3. Materials and methods

3.1. Microorganism, medium and culture conditions

C. saccharolyticus (DSM8903) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and propagated at 70 ^{o}C on DSMZ medium 640 in anaerobic 50 ml hypovials (Supelco) until OD_{600} = 0.5 cm^{-1}, corresponding to $3x10^{7}$ CFU ml^{-1}, was attained. Routine manipulations were performed in an anaerobic chamber (Bactron IV, Sheldon Manufacturing, Inc., Canada). The inoculum size was 3% (v/v). LMA and AA were purchased from Sigma – Aldrich, Germany. They were added in concentrations of 0.1 to 0.7% (w/v) to the growth medium prior to sterilization at 120 ^{o}C for 30 min. Control batches were grown without any additives.

3.2. Cell growth, viable cell counts and cell biomass

Viable cell counts were determined as colony forming units (CFU) by plating on DSMZ medium 640 solidified with 2.5% Gelrite Gellan Gum (Sigma-Aldrich, Germany) (Rakhely and Kovacs 1996). Plates were incubated at 70 ^{o}C in the anaerobic chamber for 3 to 4 days in order to develop the colonies.

Cell biomass was determined as cell dry weight (CDW). 100-ml cell suspensions were centrifuged at 15,000 x g for 15 min at 4 ^{o}C (Heraeus Biofuge Stratos, Kendro Laboratory Products GmbH, Germany) for biomass measurements. When LMA was used as an additive, centrifugation was carried out at 30 ^{o}C to avoid solidification. The pellets were washed with phosphate-buffered saline (PBS), resuspended in water and dried at 70 ^{o}C to constant weight.

3.3. Analysis of hydrogen production

35-ml aliquots of exponentially growing cultures were placed into 50-ml gas-tight hypovials fitted with rubber septa and flushed with N_2 for 10 min prior to incubation at 70 ^{o}C. H_2 production was measured by injecting 250-µl aliquots from the headspace into a gas chromatograph (6890N Network GC System, Agilent Technologies. Inc., USA) equipped with a 5Å molecular sieve column (L 30 m, I.D. 0.53 megabore, film 25 µm) and a thermal conductivity detector. The GC was calibrated with pure H_2. This method measures only the H_2 content of the gas phase; other gaseous components, e.g. CO_2 were not determined. The vials were flushed with N_2 after each daily measurement. Specific H_2 production was calculated by dividing the amount of H_2 evolved by the cells by the CFU.

3.4. Characterization of the support matrices

The natures and physical characteristics of the matrices used in the experiments are listed in Table 7.

Support matrix	Type	Particle size (mm)	Cell attachment capacity (cells support $g^{-1)}$	pH of 1% solution	Bulk density, (g cm^{-3})	Water loading capacity of matrix (ml g^{-1})
Perlite (PER)	Mineral support	0.7-2	5.2×10^6	6.9	0.07-0.10	10.0
Granular activated carbon (GAC)	Inorganic support	1.5-4	4×10^7	6.9	0.35-0.45	2.5
Pine wood shavings (PWS)	Organic support	5:2:0.5	1.2×10^7	6.8	0.15-0.18	5.0

Table 7. Characterization of support matrices.

Perlite (PER) was obtained from Corax-Bioner Corp., Hungary, granular activated carbon (GAC) was purchased from Chemviron Carbon, Belgium, and the pine wood shavings (PWS) were kindly provided by a local carpentry shop. One gram of each matrix was placed individually in a vial containing 10 ml of cell culture in the stationary phase (0.63 x 10^7 CFU ml^{-1}) and the vial was sealed and flushed with N_2 for 10 min prior to gentle shaking at 70 ^oC for 1 h. The cell-binding capacity of each immobilization matrix was determined by subtracting the initial CFU of the culture in the vial from the CFU of the supernatant (Table 7).

3.5. Cell immobilization, storage and analysis of stored cells

The matrices used in the experiments were chosen on the basis of previous reports on their use for the immobilization of bacterial and fungal cells (Dervakos and Web 1991; Razmovski and Pejin 1996Raihan et al. 1997). The supports were washed in PBS, sterilized and dried. The cell concentration was adjusted to 5% (v/v). 0.700 g of GAC, 0.175 g of PER or 0.350 g of PWS was placed in 50 ml hypovials. Freshly grown liquid culture alone (LC), or LC containing the additives LMA or AA was added to the support matrices until the maximal loading capacity of the support matrices was attained. The vials were sealed, flushed with N_2 for 10 min and stored at 42 ^oC for various periods of time. After storage fresh growth medium, with or without additive, was added anaerobically to the hypovials, which were then heated at 70 °C. LCs, grown with or without additives, were stored under the same conditions and sub-cultured in fresh growth medium to serve as controls. H_2 production was measured daily and the gas phase was replaced with N_2 after each measurement.

3.6. Protein measurement

Protein was determined by an ND-1000 Spectrophotometer V3.1, (NanoDrop Technologies, Inc., USA) at 280 nm, using bovine serum albumin as standard.

3.7. One-dimensional SDS-PAGE analysis of proteins

For the analysis of crude extract proteins, cells (0.5×10^7 CFU ml^{-1}) were harvested by centrifugation at 12,000 x g, suspended in 50mM Tris-HCl, pH 8.0 and disrupted with a French press (Thermo IEC, USA). Approximately equal amounts of protein were loaded onto each lane of 5-15% gradient sodium dodecylsulfate–polyacrylamide (SDS-PAGE) gels (Sambrook et al. 1989).

For the analysis of extracellular proteins, cells were centrifuged at 12,000 x g for 20 min and the supernatants were filtered through a cellulose acetate membrane filter (Schleicher & Schuell, Germany) with a 0.2 μm cut-off. The proteins were concentrated by the acetone precipitation method (Rosenberg 1996). SDS-PAGE was performed as above.

3.8. Total organic carbon analysis

Total organic carbon was analyzed on an Apollo 9000 Combustion TOC Analyzer (Tekmar Dohrmann, USA). Carbon in the injected sample (50 μl) was first converted to CO_2 in the combustion furnace for total organic carbon (TOC) and total carbon (TC) analysis at temperatures from 680 °C to 1000 °C. The carrier gas was nitrogen. The standards used were potassium acid phthalate and sodium bicarbonate. The water used in the analyzer was organic-free deionised water.

3.9. Total solids content analysis

Dry matter total solids as well as organic and inorganic dry weight contents were determined by the methods specified in VDI (Verein Deutcher Ingenieure, 2006). Briefly, samples were dried overnight at 70 C for determination of moisture content. Afterwards they were burnt at 550 C for 4 hours in a furnace (Nabertherm GmbH, Germany) and cooled down for 1 hour in a desiccator before measuring the weight.

Substrate	Dry matter total solids, %	Organic dry-weight content, %	Inorganic dry-weight content, %
Sorghum leaves	29.81	90.71	9.29
Sorghum stem	20.94	95.47	4.53
Sorghum juice	15.06	75.89	24.11
Sorghum juice 65 °Bx	74.01	90.92	9.08
Sugarcane bagasse	94.88	92.73	7.27
Wheat straw	95.43	90.24	9.76
Maize leaves	95.45	84.6	15.4
Pretreated maize	46.83	91.02	8.98
Silphium	41.67	78.67	21.33

Table 8. Total solids content of the biomass for H_2 production.

3.10. Determination of glucose concentration

Glucose concentration in the medium was determined by the DNSA method as described by Miller 1959.

4. Results and discussion

4. 1. Utilization of polymeric substrates by C. saccharolyticus

4.1.1. Agarose and alginic acid as a source for H_2 production by *C. saccharolyticus*

The freely suspended liquid cultures (LCs) of *C. saccharolyticus* retained hydrogen evolving capacity for up to 8 days when the initial medium contained cellobiose (CB) and yeast extract (YE). The polymers of polysaccharide derivatives LMA and AA were found to be consumed as carbon and energy sources by *C. saccharolyticus*. The polymers were tested in the concentration range 0.1-0.7% (w/v). In almost all the experiments, 0.1% (w/v) cellobiose (CB) was used, in addition to the standard YE, as a readily metabolized carbon source to facilitate the initial cell growth. Propagation in the presence of LMA and AA caused elevated H_2 evolution (Fig. 10).

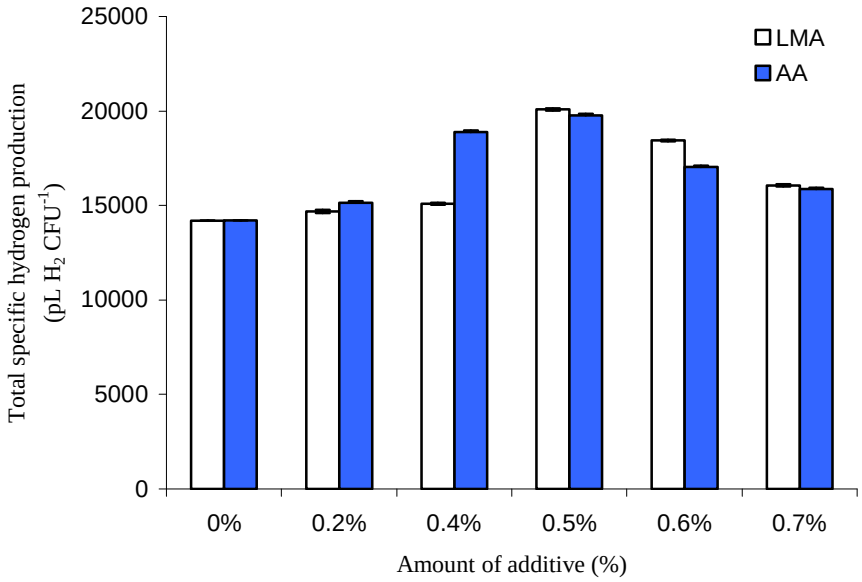


Figure 10. Total H_2 production by liquid cultures grown on cellobiose (CB) and CB with low-melting agarose (LMA) or alginic acid (AA) added in various concentrations.

Figure 11 suggests that the improvement was due, at least in part, to an increased biomass production.

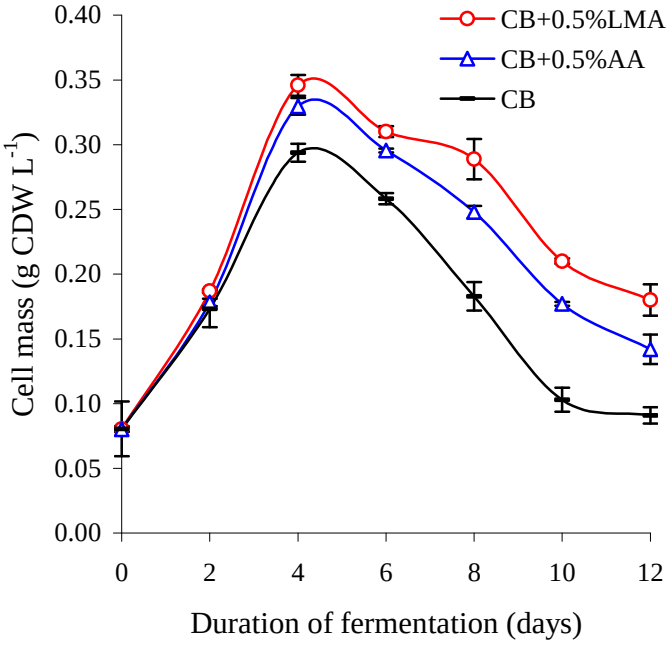


Figure 11. Biomass production of *C. saccharolyticus* grown on cellobiose (CB) and CB with 0.5% agarose (CB + 0.5% LMA) or 0.5% alginic acid (CB + 0.5% AA).

The additive concentration corresponding to the highest H_2 production was 0.5% (w/v) for both polymers (Fig 10). The H_2-evolving capacity of the polymer-fed cells increased 1.37- and 1.92-fold relative to the LC controls (see also Table 9, rows 1-3).

Hydrogen production data (pL H_2 CFU^{-1})												
Treatment	Days of storage											
	0	1	2	3	5	8	14	18	22	26	30	Sum
1. LC	14,211	14,199	14,184	13,765	13,585	11,628	0	0	0	0	0	81,573
2. LC+LMA	20,726	20,572	20,196	19,774	17,493	16,585	14,529	9,775	9,321	7,800	0	156,771
3. LC+AA	19,681	19,198	19,078	18,841	17,447	14,093	4,541	1,696	0	0	0	114,575
4. GAC	14,211	14,210	14,127	13,955	13,585	12,293	11,138	10,139	8,689	0	0	112,348
5. GAC+LMA	20,503	20,503	20,373	20,245	19,326	18,805	16,403	18,525	14,793	13,515	10,229	193,218
6. GAC+AA	19,681	19,188	19,121	19,065	17,657	14,795	5,498	2,673	0	0	0	117,678
7. PWS	75,110	75,110	74,813	74,813	63,492	54,051	42,995	32,618	26,749	16,763	11,801	548,315
8. PWS+LMA	80,515	80,515	80,116	80,116	69,084	59,768	47,638	36,480	30,087	19,300	13,482	597,101
9. PWS+AA	80,579	80,108	79,700	79,880	68,381	56,501	37,450	25,261	26,751	16,760	11,800	563,171
10. PER	14,211	14,169	14,160	13,849	12,873	12,053	11,244	9,833	0	0	0	102,392
11. PER+LMA	20,726	20,706	20,455	19,549	15,952	13,564	9,453	6,263	4,318	0	0	131,052
12. PER+AA	19,681	19,202	19,102	18,973	17,597	14,105	4,540	1,690	0	0	0	114,890
LC, liquid culture; LMA, 0.5% low melting agarose; AA, 0.5% alginic acid; GAC, granular activated carbon; PER, perlite; PWS, pine wood shavings.												

Table 9. Hydrogen production data

Neither polymer supported growth in the absence of CB and YE. A different picture emerged when PWS were used as an additive polymeric substrate. In the presence of PWS, *C. saccharolyticus* needed only a small amount of YE to start growth and then the culture maintained its biological activity for almost 2 months producing a total of 14.3 times more H_2 than the controls without PWS (Fig. 12), which produced H_2 only during the consumption of the YE in 6 days.

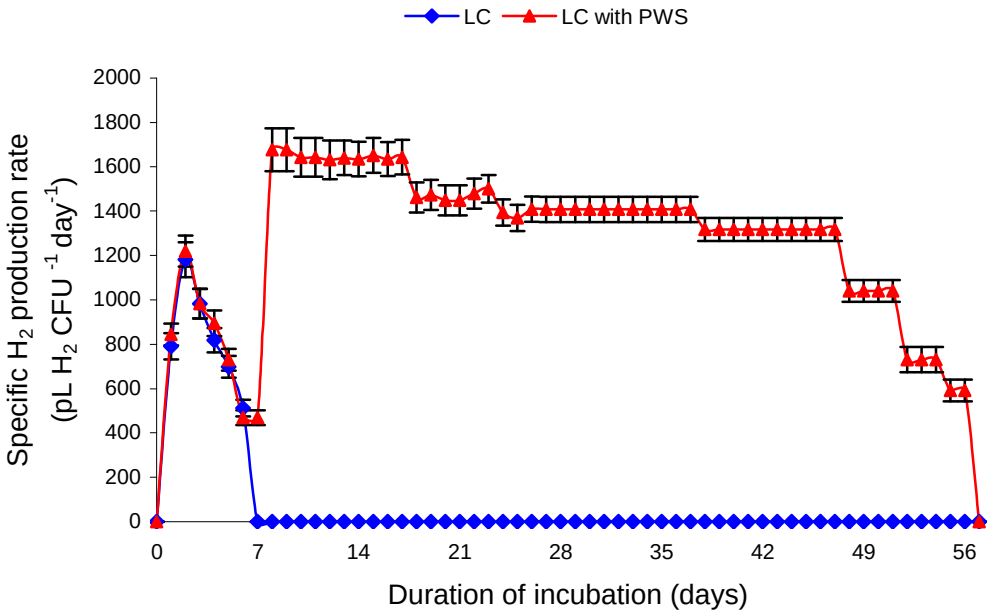


Figure 12. Daily H_2 production by a freely suspended liquid culture grown on DSMZ Medium 640 without cellobiose (LC) and with 50 mg pine wood shavings (LC with PWS).

The addition of PWS led to increased total organic carbon content in the medium (Fig. 13), indicating that soluble carbonaceous compounds were released from PWS by the cells.

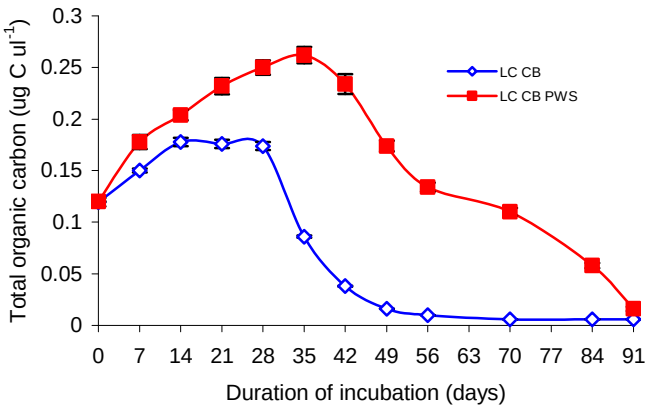


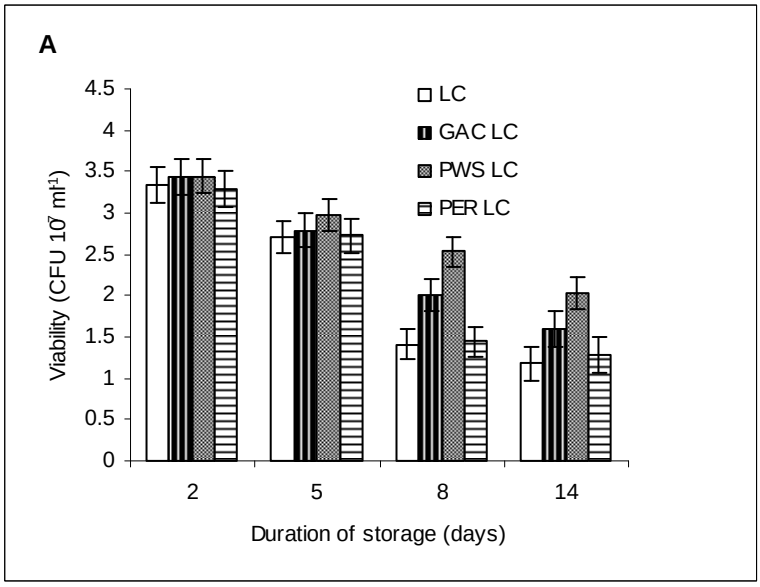
Figure 13. Changes of total soluble organic carbon released in time by liquid cultures grown on cellobiose as sole carbon source (LC CB) and on cellobiose and pine wood shavings (LC CB PWS).

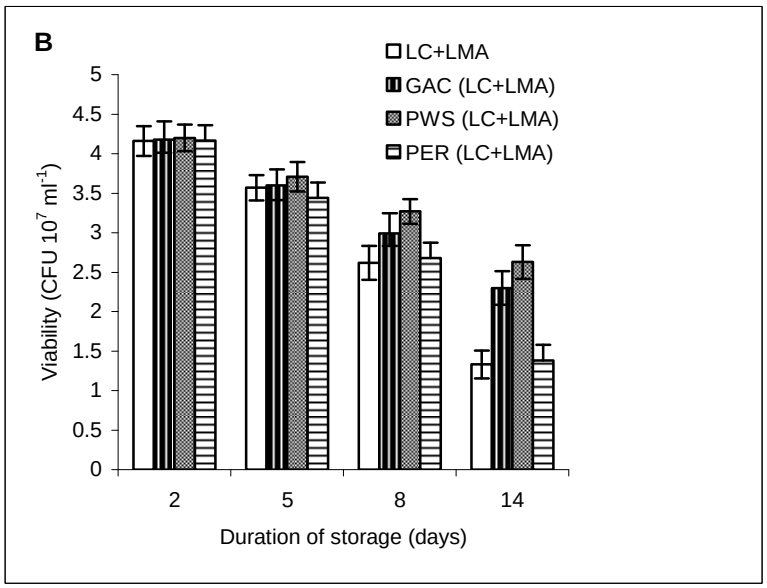
Under these experimental conditions about 1% of the total mass of PWS was converted to H_2. LMA and AA did not contribute significantly to the H_2 production from PWS (Table 9, rows 7-9). Relative to the LC, *C. saccharolyticus* cells on PWS displayed a remarkable increase in H_2 production within 30 days, i.e. 6.72-fold and there was 3.80- and 5.21-fold increase for the LMA or AA containing LC samples, respectively (Table 9, rows 1-3 and 7-9).

4.1.2. Anaerobic storage of *C. saccharolyticus* and hydrogen production by stored cells

The observation of wood utilization by *C. saccharolyticus* prompted us to study the survival of LCs and immobilized *C. saccharolyticus* cells during anaerobic storage. Aerobic exposure of the cells for 5 hours caused a drop of cell activity with about 50%, and 12 hours aerial exposure inactivated the cells irreversibly (data not shown). Cells were stored anaerobically at 30 ^{o}C, 37 ^{o}C or 42 ^{o}C for various periods of time and subsequently cultured in fresh growth medium with or without additives in order to determine optimal storage conditions. The viability and H_2-producing properties of the cells were best preserved at a storage temperature of 42 ^{o}C. Storage at 30^{o}C led to complete inactivation.

Accordingly, the LCs were stored anaerobically at 42 ^{o}C, then transferred into fresh medium containing LMA or AA and incubated at 70 ^{o}C with analysis for daily H_2 production and viability (Fig. 14a-c) until H_2 production ceased.





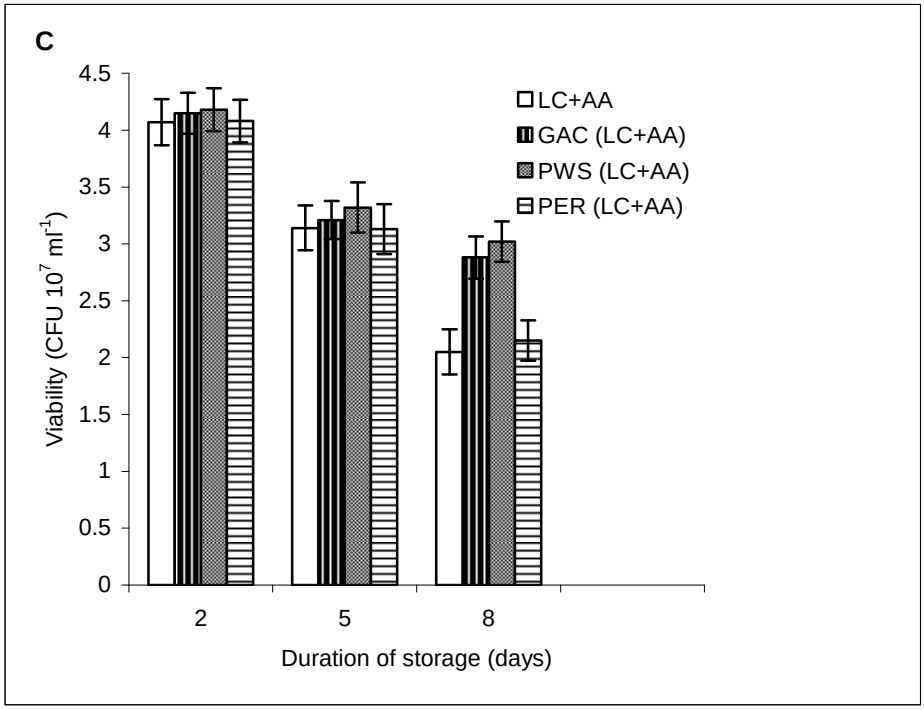


Figure 14. Viability of stored *C. saccharolyticus* on matrices on the second day of regeneration in fresh medium; (A) LC; (B) LC + LMA; (C) LC + AA. Cells were stored at 42 ºC as given in Materials and Methods, data corresponding to 2, 5, 8, and 14 days of storage are plotted. Values are the average of three measurements and are expressed in (CFU ml^{-1}) units.

Controls were treated identically, but without the additives. Storage for up to 8 days under these conditions did not greatly impair the cell viability and H_2-production ability (Fig 14 and Table 9). Following this storage time, the shorter the storage time, the longer the duration of active H_2 production. Longer storage times were associated with a progressive loss of viability (Fig. 14). Cells grown in a medium supplemented with LMA retained their viability and H_2-production capacity longer than cells grown in AA-containing medium; the cells without additives exhibited the poorest survival (Fig. 15).

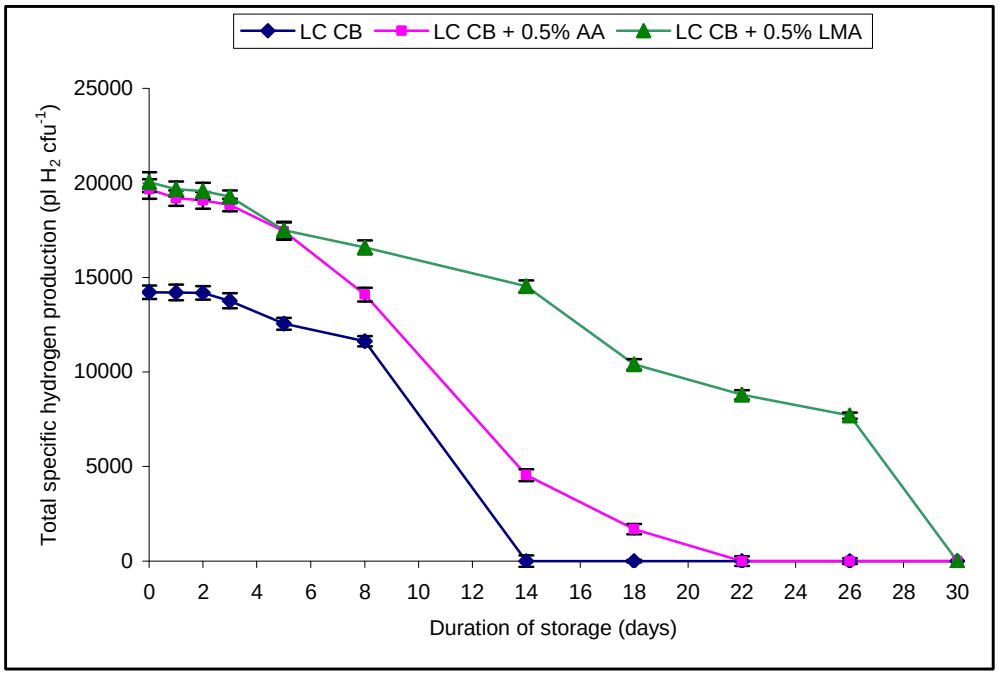


Figure 15. Total H_2 production rates of cells stored for various times and then cultured in standard liquid medium (LC CB) with 0.5% alginic acid (LC CB + 0.5% AA) or 0.5% agarose (LC CB + 0.5% LMA).

In LCs in the presence of LMA *C. saccharolyticus* still retained metabolic activity after 26 days of storage, which was 18 days longer than for the control (Fig. 15). AA contributed moderately to the preservation of cell viability and H_2-production capacity (Figs. 14 and 15). These experiments demonstrated that LMA supported the survival of stagnating *C. saccharolyticus* cells and served as a moderately good substrate for H_2 production, while AA was less efficient.

The potentiating effects of LMA, AA and PWS on H_2 evolution by *C. saccharolyticus* were demonstrated. The LMA was presumably degraded by agarases, known in some bacteria (Van der Meulen and Harder 1975; Kendall and Cullum 1984; Potin et al. 1993; Sugano et al. 1993; 1994; Ha et al. 1997; Vera et al. 1998) but representing only a few phyla and classes. Such isolates inhabit seawater, salt marshes, fresh water and soils (Michel et al. 2006) and the agarases they produce are generally classified into two groups, according to their mode of action, i.e. α-agarase and β-agarase, which hydrolyze the agarose α-1,3 and β-1,4 linkages, respectively. With the exception of the enzyme from *Alteromonas agaralytica* (Ohta et al. 2004), all known agarases hydrolyze the β-1,4 linkage of agarose, yielding oligosaccharides

in the series related to neoagarobiose [O-3,6-anhydro-α-L-galactopyranosyl-(1→3)-D-galactose]. It should be noted that galactose is an excellent carbon source for growth of *C. saccharalyticus*, better than cellobiose (our observation, data not shown).

There have been no reports on agarolytic extreme thermophilic bacteria. Our studies revealed that LMA is a good substrate for H_2 production (Fig. 15) and increases the viability of *C. saccharolyticus* cells upon storage (Fig. 14b and Table 9). These are strong indications of the presence of an agarose-degrading enzyme in this bacterium. The addition of LMA to the growth medium of *C. saccharalyticus* also caused an elevated protein content in the extracellular protein fraction, and a number of new bands on an 8% SDS-PAGE gel (data not shown), which is in line with the assumption that the tentative agarase of *C. saccharolyticus* does exist and is an extracellular enzyme. Additional indirect evidence is the observation that reducing sugars accumulate in the extracellular fraction only when the cells are grown on agarose, a polymer of D-galactose, a reducing sugar (data not shown). Such activity was not detected in the whole cell fraction or on cell fractions grown without LMA.

Alginates, which have wide-ranging commercial applications (Velings and Mestdagh 1995; Ertesvag and Valla 1998; Edwards-Levy and Levy 1999) are found in great abundance in brown seaweeds and in two families of heterotrophic bacteria, the *Pseudomonadaceae* and the *Azotobacteriaceae* (Preiss and Ashwell 1962; Wong et al. 2000). Alginate degradation, catalyzed by alginate lyases (alginases and alginate depolymerases) isolated from numerous microorganisms (Wong et al. 2000) involves a β-elimination mechanism that has yet to be fully elucidated. The alginases have been characterized as either mannuronate or guluronate lyases. Alginases synthesized by anaerobic extremophiles have not yet been reported and due to the complex and undefined structures of alginate and its purified derivative AA, it is difficult to assume which type is active in *C. saccharolyticus*. This extremophile degrades not only alginic acid, but also sodium alginate. The commercial alginate product was heterogeneous, it might have contained mono-, di- or oligomers, which could serve as a substrate for the cells. Therefore the commercial alginate was dialyzed overnight at 4^{o}C and freeze-dried in order to get rid of the monomeric and oligomeric contaminations. Hydrogen production and the degradation kinetics were very similar to that of the AA (data not shown). Interestingly, both alginate and AA proved suitable as polymeric substrates for *C. saccharolyticus* cells only when the cells were stored anaerobically at 42 ºC for not more than 8 days in the standard growth medium (Fig. 14c). Older cultures lost the ability to degrade AA, even after replacement of the growth medium with a fresh one. The explanation of this

phenomenon and the nature of the change in metabolic status of the cells are unknown, but the effect was observed systematically in both LCs and immobilized systems, regardless of the immobilization matrix used except for PWS (Fig. 15 and Table 9, rows 3 and 9). Analysis of the protein pattern of alginate-treated cells was not possible by SDS-PAGE in consequence of the strong interference of the reagents used with even extremely minor amounts of alginate left on extensively washed cells.

The cellulolytic activity of anaerobic extreme thermophiles has been known for some time (Bergquist et al. 2000; Sissons et al. 1987; Hudson et al. 1990; Hudson et al. 1991), and *C. saccharolyticus* was originally described as a cellulolytic bacterium (Rainey et al. 1994) though this property has not been studied in detail subsequently. Soft wood shavings from the pine are degraded by the extremophilic anaerobe in the absence of any other carbon source in the growth medium. Attempts to hydrolyze wood shavings from oak trees (hard wood) were not successful, presumably because of the distinct composition of this lignocellulosic raw material (Iranmahboob et al. 2002). The bacterial hydrolysis of lignocelluloses is usually preceded by physical and/or chemical pretreatment in order to break up the well-organized and recalcitrant structure (Van Niel et al. 2003; Kadar et al. 2004; Iranmahboob et al. 2002. *C. saccharolyticus* can apparently use soft wood as carbon and energy sources for biohydrogen production without any pretreatment (Fig. 12). It must perform the hydrolysis via its own cellulolytic apparatus, probably solubilizing the hemicellulose fraction of the wood, which might explain the increased total organic carbon content in the growth medium (Fig. 13) and considerably elevated H_2 production (Fig 12 and Table 9, rows 1-3 and 7-9). The extracellular protein concentration of the spent medium after growth on CB and PWS as carbon sources was higher than that of a growth medium containing CB alone as carbon source. This may suggest the presence of induced (hemi)cellulases in the extracellular space (data not shown). Recent reports on the architecture of the cellulolytic enzymes of certain anaerobes indicate that these enzymes are multiprotein complexes consisting of many subunits, e.g. the cellulosome of Clostridia (Newcomb et al. 2007). We plan further analysis on the molecular structure of the cellulases in *C. saccharolyticus*, which is phylogenetically related to Clostridia.

4.1.3. Immobilization studies

The use of various zeolites (Na^+- and NH_4^+-exchangers, silicate powders) as immobilization matrices did not support the survival of *C. saccharolyticus* (data not shown). Immobilization of *C. saccharolyticus* on GAC, PER or PWS had a beneficial effect on the survival of the cells stored at 42 ^oC relative to the LCs, in the sequence PER<GAC<PWS (Table 8). In the following experiments, the effects of using these immobilization matrices alone or in combination with LMA and AA on the survival during storage and H_2 production from CB or from CB supplemented with LMA or AA were studied. The positive effect of LMA on the stored immobilized cells was observed after the cultures had consumed the YE and CB, i.e. after about 8-13 days of storage. AA displayed the same modest effect as seen before: cells which had been stored for up to 8 days produced significantly elevated amounts of H_2 after replacement of the medium with a fresh one containing YE, CB and AA, but the H_2 production of cells stored for a longer period of time rapidly decreased when the same substrates were applied (Table 9, rows 3, 6, 9, 12). The total H_2 production of GAC-immobilized and LMA-treated stored cells was 2.37 times higher than that of stored LMA supplemented LCs (Table 9, rows 2 and 5). For perlite the raise was 1.60-fold (Table 9, rows 8 and 10).

C. saccharolyticus immobilized on PWS displayed an outstandingly higher H_2 productivity than that of the LCs, with improvements both in storage survival and H_2-evolving capacity. It should be noted that PWS-immobilized *C. saccharolyticus* did not need CB to survive and it retained its biological H_2-production activity for up to 56 days (Fig. 12). The total H_2 produced in these samples during the first 30 days was 6.72-fold higher than the control samples without PWS, clearly indicating the most significant contribution of PWS (Table 9, rows 1-3 and 7-9). PWS in combination with LMA resulted in a 7.32-fold intensification in the H_2 evolution relative to LC. It should be emphasized that shavings of hardwood, e.g. oak, behaved completely differently. In the presence of hard wood shavings neither the stabilization of *C. saccharolyticus* cells upon storage nor an enhanced H_2-evolving capacity was observed (data not shown).

In any practical application of *C. saccharolyticus* in biohydrogen producing systems, optimal working conditions for a high production yield and a long cell lifetime are critical. Accordingly, LCs were compared with *C. saccharolyticus* cells immobilized on various

matrices from two aspects. First, we tested how long cells remained viable upon storage at 42 ºC without replenishment of their growth medium. Second, the regeneration of the H_2-evolving capacity of stored cells was followed. *C. saccharolyticus* cannot grow at 42 ºC, and lower storage temperatures were found to be deleterious for the survival of the cells. On each day, throughout the storage experiment, a set of vials was opened and analyzed for CFU and H_2 productivity and the recovery of H_2 production was followed in fresh medium at 70 ºC until H_2 evolution ceased completely due to the exhaustion of the substrates in the growth medium. The growth medium always contained a small amount of YE, which was required for starting the culture, and in many cases CB was also added. In some experiments this carbon source was supplemented with LMA, AA, or PWS. LMA was used for practical reasons as this type of agarose does not harden at room temperature.

NH_4^+- and Na^+-exchanger zeolites and mesozeolites (e.g. silicate powder) proved to be poor immobilization supports (data not shown). Granulated activated carbon (GAC), perlite (PER) and pine wood shavings (PWS), however, were good support matrices and had no toxic effects on *C. saccharolyticus*. GAC performed better than PER. The experiments showed that the matrix-related effects were advantageous for the cell physiology, prolonging the viability of stored cells by about 10-12 days relative to LCs (Table 9 and Fig. 14). GAC presumably allows a favourable distribution of the cells inside the pores and/or supplies an active surface to maintain a fraction of the cell population with metabolic activity even after 30 days of storage (Table 8). The regulation of the cellular metabolism of cells immobilized on solid supports might be influenced by physical interactions at the cell surface, which may enhance and/or reduce the cellular metabolism or some parts of it (Karel et al. 1985; Van Loosdrecht et al. 1990). It has also been suggested, though not experimentally proven, that GAC contains structured water, and the close vicinity of the cells may constitute an environment with reduced water activity (Mattiasson and Hahn-Hagerdal 1982). The experimental data on cells immobilized on PER or GAC confirmed that they did not undergo growth during storage. Hence, the living cells could be regarded as resting or starving cells, and the number of dead cells obviously increased in time (Fig. 14).

A completely different picture emerged from the experimental data on *C. saccharolyticus* cells immobilized on PWS, which was shown to be the best support properties for *C. saccharolyticus* immobilization. Under anaerobic storage conditions in the presence of PWS the cells survived for up to 56 days at 42 ºC. This is excellent endurance as compared with the 14-26 days on the other matrices, or 8-10 days in LC (Table 9 and Fig. 14). Besides providing a superior surface for cell attachment, PWS also supplied carbon and

energy for the immobilized cells. Although no detectable cells growth was observed during storage, a minuscule fraction of this immobilization matrix could have been used by the cells for maintenance under storage conditions thereby improving their survival. This is corroborated by the extended H_2 production when only PWS were present as carbon source in the system (Fig. 12). The addition of CB facilitated cell growth and H_2 production, but it was not essential, as revealed by the fact that *C. saccharolyticus* could grow and produce H_2 on PWS as a sole carbon source.

Alternative polymeric carbon sources such as LMA and AA were utilized by both LC and immobilized cells. This extends the range of potential substrates for biohydrogen production by *C. saccharolyticus* and demonstrates the metabolic versatility of this extreme thermophile. The data presented in this paper suggest that *C. saccharolyticus* is best used for biohydrogen production in an immobilized form on a suitable matrix as both its endurance and biological activity are affected positively by immobilization (Table 9). From practical point of view it is particularly promising that *C. saccharolyticus* can efficiently utilize various polymeric substrates for biohydrogen production making this extremophile an excellent candidate for use in large scale operations as well. Experiments have been performed to test other agricultural waste sources as possible substrate for economically feasible biohydrogen generation by this system.

4.1.4. Agarose-induced changes in the protein pattern

In an attempt to acquire preliminary experimental data for characterization of the agarase synthesized by *C. saccharolyticus*, 5-15% gradient SDS-PAGE of a crude bacterial extract was performed. The protein pattern of LMA-treated cells differed from that of cells grown in medium with CB as sole carbon source. Some new bands appeared in the range 35-40 kDa, whereas those at 48 kDa and 100 kDa disappeared (Fig. 16). New enzyme activities were therefore induced in the presence of added LMA. Further studies are needed to identify the proteins involved in this process. A preliminary BLAST search in the genomic DNA data base did not reveal specific agarase or alginase sequences.

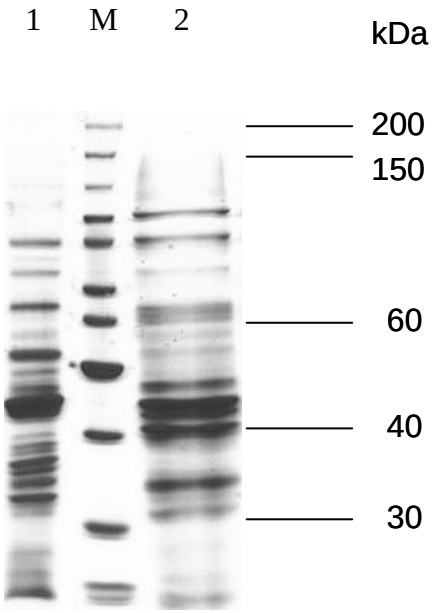


Figure 16. SDS PAGE of 5-15% gradient gel of *C. saccharolyticus* crude extract.
Lane 1 – 0.5% agarose-amended *C. saccharolyticus* cells; M – marker Fermentas #0661 (10kDa – 200 kDa), lane 2 – control *C. saccharolyticus* cells (LC CB).

4.2. Utilization of energy plants as a substrate for H_2 production by C. saccharolyticus

All plants and their derivatives, used in the experiments, were dried before use, homogenized and added in different percentage to the growth medium prior sterilization. No other carbohydrate source was used in the growth medium. For comparative studies, 0.1% cellobiose was used in the standard DSMZ 640 medium as the only energy and carbon source. Wheat straw, sweet sorghum and its concentrated juice derivatives, maize leaves, silphium and bagasse were shown to be utilized as a carbon and energy source by *C. saccharolyticus*. The biomass was added in the concentration range 0.1-2.5% (w/v) to establish the optimal percentage, resulting in the highest H_2 production (Table 10 and 11).

Substrate	Concentration, % (w/v)										
	0.1	0.25	0.5	0.6	0.7	0.8	0.9	1.	1.1	1.5	2
WST	n. d.	n. d.	16,358	n. d.	n. d.	n. d.	35,484	**36,692**	34,861	33,505	n. d.
UML	6,363	n. d.	n. d.	n. d.	6,202	6,421	**6,658**	6,465	n. d.	n. d.	n. d.
PML	2,386	6,363	7,231	**8,201**	8,101	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
STR		n. d.	5,610	n. d.	n. d.	n. d.	n. d.	**5,912**	n. d.	5,425	5,243

10A.

Substrate	Concentration, % (w/v)												
	0.25	0.5	0.7	0.9	1	1.1	1.5	1.8	1.9	2	2.1	2.2	2.5
SSP	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	13,581	27,975	n. d.	**28,743**	n. d.	27,050	n. d.
BGS	n. d.	1,820	2,731	4,096	**5,591**	4,538	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

10B.

Substrate	Concentration, % (w/v)												
	0.01	0.03	0.05	0.07	0.1	0.15	0.19	0.2	0.21	0.25			
SSC	2,311	3,466	3,627	**3,779**	2,993	n. d.	n. d.	n. d.	n. d.	n.d.			
SSJ	n. d.	n. d.	n. d.	n. d.	1,130	4,754	5,085	**5,123**	5,085	4,886			

10C.

Table 10. Total specific hydrogen production rates (pL H_2 CFU^{-1}) of various biomass sources.

Substrate	THPR (pL H_2 CFU^{-1})	H_2 productivity (L H_2 kg^{-1} dry biomass)	H_2 yield (mol mol^{-1} glucose consumed)
SSC**	3,779	131.93	2.98
SSJ**	5,123	55.08	2.63
WST*	36,692	44.68	3.80
PML*	8,201	38.14	3.67
SSP*	28,743	30.17	1.75
BGS*	5,591	18.21	2.30
UML*	6,658	15.33	1.80
STR*	5,912	8.14	0.48
PWS*	71,253	4.32	0.57

Table 11. Total hydrogen production rate (THPR), productivity and yield of *C. saccharolyticus* grown on dry biomass. (*) dry solid biomass; (**) liquid biomass.

4.2.1. Wheat straw as a carbon source for H_2 production

Propagation of *C. saccharolyticus* in the presence of wheat straw in concentrations from 0.5% to 1.5% (w/v) brought about elevated H_2 evolution (Fig. 17). Maximal production rate of H_2 was observed when 1% wheat straw was used as a carbon source. Concentrations higher than 1% caused a reduction in H_2 evolution probably due to substrate inhibition. Hydrogen production began on the first day and maintained at high level for 11 days, when the optimal performance was reached. The H_2 evolution lasted for 45 days, with a rapid decrease from the 11th to the 23rd day, after which a low constant production rate was maintained. The H_2 evolution ceased on the 45th day, presumably due to the unavailability of any fermentable substrate left.

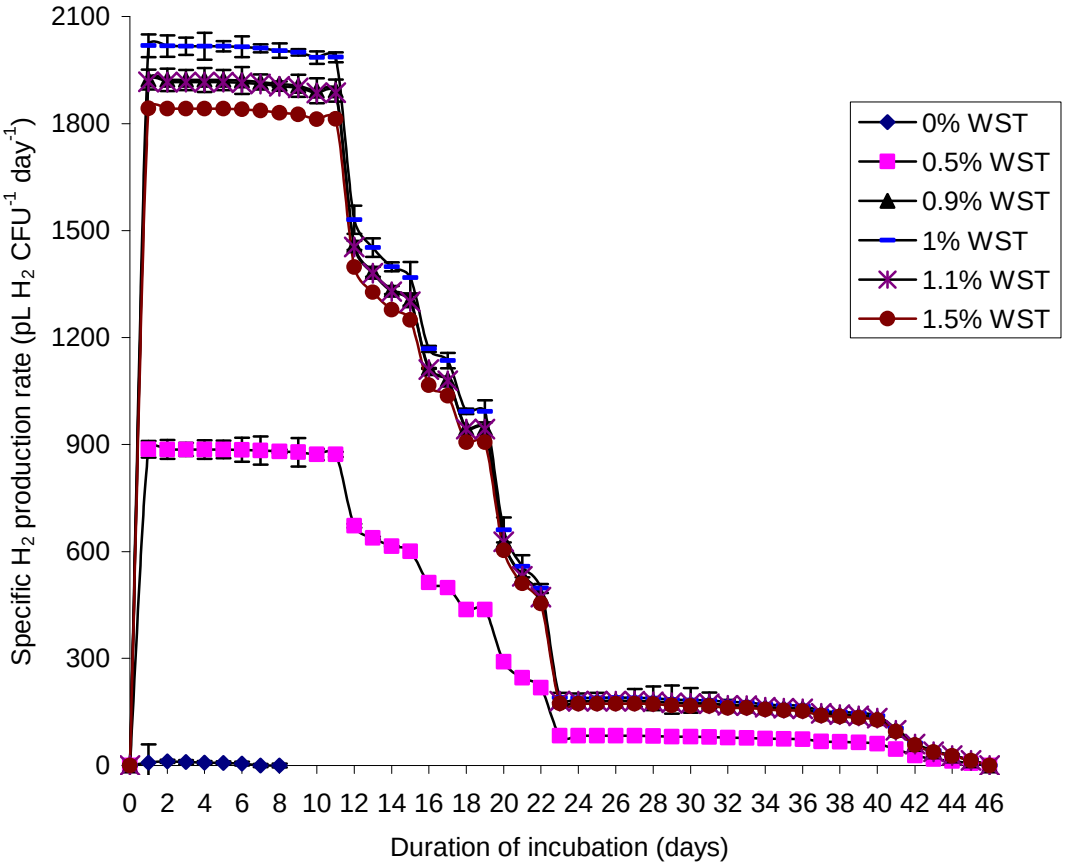


Figure 17. Daily hydrogen production rate by *C. saccharolyticus* grown on wheat straw as a carbon source

The H_2 production rate was almost twice as much (1.94-fold higher) when 0.5% (w/v) wood shavings were used as a sole energy and carbon source relative to the wheat straw (Fig. 12 and Fig. 17, Table 10 and 11). The higher H_2 production rate of PWS was coupled with a prolonged fermentation time of 56 days, 11 days longer than the fermentation on wheat straw. However, wheat straw yielded about 6.7-fold higher amount of H_2 for a unit of consumed glucose and the H_2 productivity was about 10-fold higher (Table 11), indicating that the lignocellulosic composition of the wheat straw was more easily accessible for enzymatic hydrolysis by *C. saccharolyticus* than PWS under the same fermentation conditions.

Fan et al. 2006 reported on a maximal hydrogen yield of 68 mL H_2/g total solids when HCl pretreated wheat straw wastes have been degraded anaerobically by cow dung compost at 36 ºC and that yield was 136-fold higher relative to the raw wheat straw. In our study we report on a maximal hydrogen yield of 50 mL H_2/g total solids when raw wheat straw was used without any pretreatment by *C. saccharolyticus*, indicating that the extremophile was about 100-fold more efficient than the mesophilic mixed enrichment used by Fan et al. 2006. On the other hand, Pan et al. 2008 showed that activated sludge of paper mill was about 2.6-fold more efficient in producing biohydrogen from wheat brans in comparison to *C. saccharolyticus*, but again they have employed time and energy consuming pretreatment processes.

So far there are no reports in the literature on biohydrogen production by an extremophile, growing on wheat straw as an energy and carbon source without any pretreatment. It meets the increased needs for alternative energy resources. In that respect, wheat straw, being a cheap, widely available and renewable resource, can be considered advantageous for H_2 production over other alternatives like energy-intensive chemical processes (pyrolysis, gasification), or low-efficient bio-photolysis processes.

4.2.2. Sweet sorghum plant and its extracts as carbon source for H_2 production

Fresh sorghum plants (*Sorghum bicolor)* were homogenized, air dried and used as an energy and carbon source in different concentrations, yielding the highest H_2 production when 2 % (w/v) was applied (Fig. 18). Concentrations higher than 2% caused a decrease in H_2 evolution. At the optimal concentration, *C. saccharolyticus*, grown on sweet sorghum produced H_2 for 34 days, yielding only about 22% less H_2 relative to the wheat straw

substrate (Fig. 17 and Fig. 18, Table 10 and 11). The hydrogen production rate varied very little, with a decrease from 1,300 pL H_2 CFU^{-1} day^{-1} to 1,000 pL H_2 CFU^{-1} day^{-1} in 23 days, which may indicate a more homogeneous distribution of the accessible substrate compared to the wheat straw. A fast decrease in H_2 production followed due to depletion of the available substrate (Fig. 18) and that was coupled with a drop of the cell viability (data not shown).

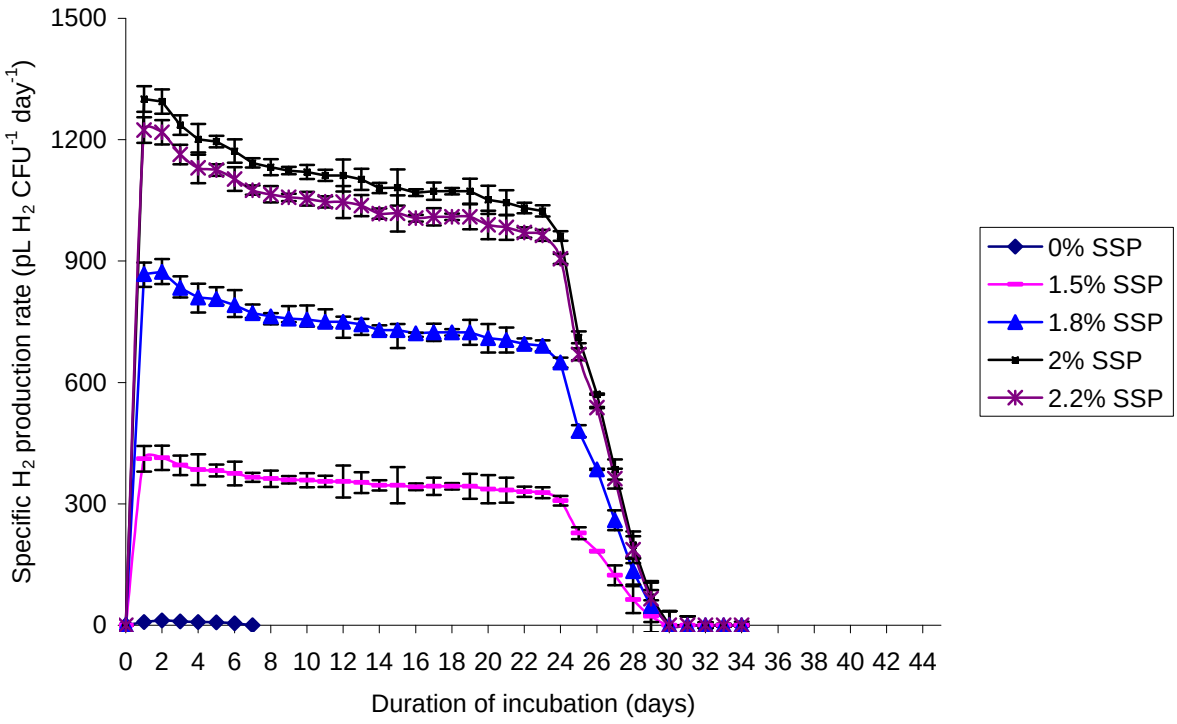


Figure 18. Daily hydrogen production by *C. saccharolyticus* grown on sweet sorghum as a carbon source

In a different experiment, sweet sorghum plant was stripped into leaves and stems and water extraction at 30 °C of the available sugars was performed according to the method of Ntaikou et al. 2008. The extract, containing the readily extractable sugars accounted for 7.5 % of the overall H_2 production, the leaves contributed by 53.2% and the stems by 39.3%. The sum of H_2 evolution, coming from the three treatments equalled that one of the homogenized whole plants (data not shown).

Reports on the use of sweet sorghum plants for H_2 production started to emerge in the literature recently. Ntaikou et al. 2008 used mesophilic pure culture of *Ruminococcus albus* to

produce hydrogen from wet sweet sorghum biomass. They reported H_2 productivity of 58 L H_2 kg^{-1} wet sorghum biomass which is in a good agreement with our studies of 30.17 L H_2 kg^{-1} dry sorghum biomass (Table 11) and H_2 yield of 2.5 mol H_2 mol^{-1} consumed glucose for sorghum residues, which is consistent with our yield of 1.75 mol H_2 mol^{-1} consumed glucose. Our data fit very well to the H_2 yield, reported by Martinez-Perez et al. 2007, who indicated 1.7 mol H_2/mol hexose converted in sweet sorghum in anaerobic digesters.

We report for the first time on H_2 production by an extremophile using sweet sorghum plants as a growth substrate. We have proven that H_2 production from sweet sorghum by the extremophile *C. saccharolyticus* without any pretreatment of the substrate is possible and stable for a period of 24 days under batch fermentation conditions, with an average production rate of 1000 pL H_2 CFU^{-1} day^{-1}, the second best among the other utilized substrates (Figure 18). In terms of H_2 productivity, sweet sorghum comes in the fifth place as the most suitable H_2 substrate after its derivatives SSC and SSJ, wheat straw and pretreated maize leaves (Table 11).

Preliminary studies have been carried out by Claassen et al. 2004 and Antonopoulou et al. 2008 on biohydrogen production from sweet sorghum derivatives like extracts and bagasse. We also demonstrated that *C. saccharolyticus* was able to produce H_2 from SSJ and SSC (Table 10, Figures 19 and 20). The highest H_2 production for SSJ was reached when 0.2% SSJ was used with concurring batch fermentation of 34 days. For SSC the optimal percentage for H_2 production was 0.07% and the fermentation was 10 days shorter. Claassen et al. reported on 4 mol H_2/mol C_6 which is close to our data of 3 mol H_2/mol glucose when *C. saccharolyticus* was grown on SSJ (Table 11). Antonopoulou et al. 2008 reported on H_2 yield of 0.86 mol H_2/mol glucose consumed which is half of the value for SSP and over 3-fold lower for SSJ and SSC relative to our studies. Their H_2 productivity for SSJ was about 5-fold lower than our results (Table 11).

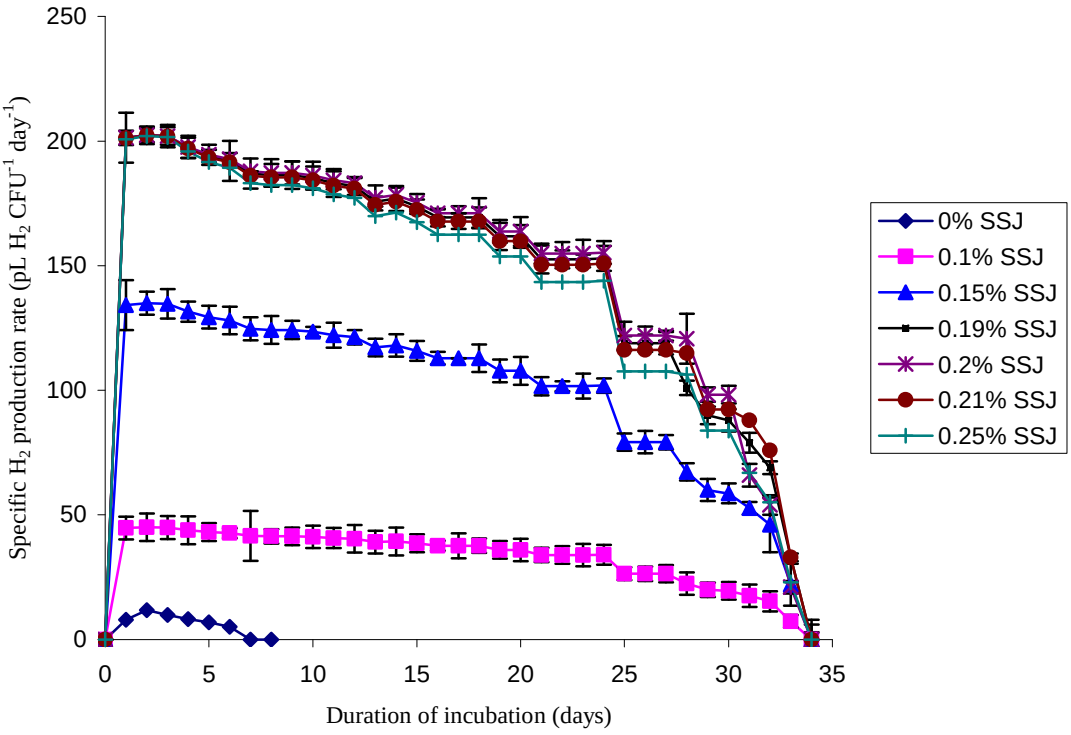


Figure 19. Daily hydrogen production by *C. saccharolyticus* grown on sorghum juice as carbon source

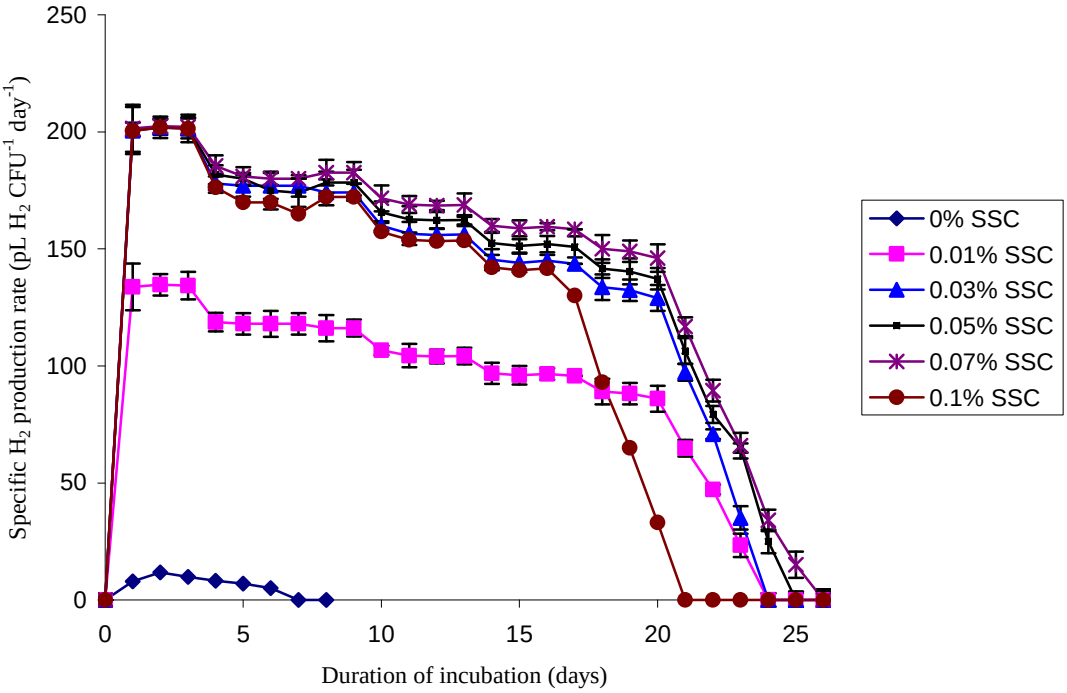


Figure 20. Daily hydrogen production by *C. saccharolyticus* grown on sweet sorghum concentrate 65 ^{o}Bx as carbon source.

4.2.3. Maize leaves as carbon source for H_2 production

The use of maize leaves for H_2 production by *C. saccharolyticus* was shown to be successful. The highest H_2 rate was reached at 0.9% (w/v) and the fermentation lasted for 48 days (Fig. 21). The hydrogen production rate was about 11-fold lower compared to wood shavings and about 5.5-fold lower than that of the wheat straw fermentation. In an attempt to improve the yield of H_2 production, maize leaves were enzymatically pretreated with *Bacillus amyloliquefaciens*. 10% biomass (w/v) was added to 50 mM phosphate buffer and incubated with *B. amyloliquefaciens* at 30 °C on a rotary shaker for 3 days. The supernatant was removed and the remaining biomass was dried and used for H_2 fermentation by *C. saccharolyticus*. The H_2 rate improved by 23% when 0.6% pretreated maize leaves were used and the fermentation was prolonged with 4 days (Fig. 22, Table 10). Addition of more than 0.6% pretreated maize biomass decreased the H_2 production rate. *C. saccharolyticus*, grown on PML, yielded about 2.1-fold higher H_2 in comparison to the H_2 yield on sweet sorghum, and had 1.3-fold higher H_2 productivity related to SSP (Tables 10 and 11). The PML H_2 yield was the second best among the tested substrates after WST, while that of UML ranked 6th (Table 11). Our results for H_2 yield for UML are consistent with the data, reported by Martinez-Perez et al. 2007, who indicated 1.9 mol H_2/mol hexose relative to 1.8 mol H_2/mol glucose in our studies (Table 11). Furthermore, by pretreating the maize leaves we have improved the H_2 yield, reaching 3.6 mol H_2/mol consumed glucose and we have obtained H_2 yield of 81.5 ml H_2/g dry matter for PML, which is higher by 31% compared to the H_2 yield of Kayazze et al. 2008 (62.4 ml H_2/g dry matter). UPM yielded only 16 ml H_2/g dry matter, almost 4-fold lower than the yield reported by Kayazze et al. 2008. The use of maize leaves or fodder for H_2 production has been acknowledged just recently, applying anaerobic mixed microflora. Kyazze et al. 2008 concluded that the majority of the complex structural carbohydrates of fodder maize were not accessible for metabolism by the anaerobic mixed culture judging by the low increase of the neutral and acid detergent fibers, hemicellulose and holocellulose of the fodder maize before and after the fermentation. That might be one of the reasons for the lower H_2 yileds in comparison to wheat straw.

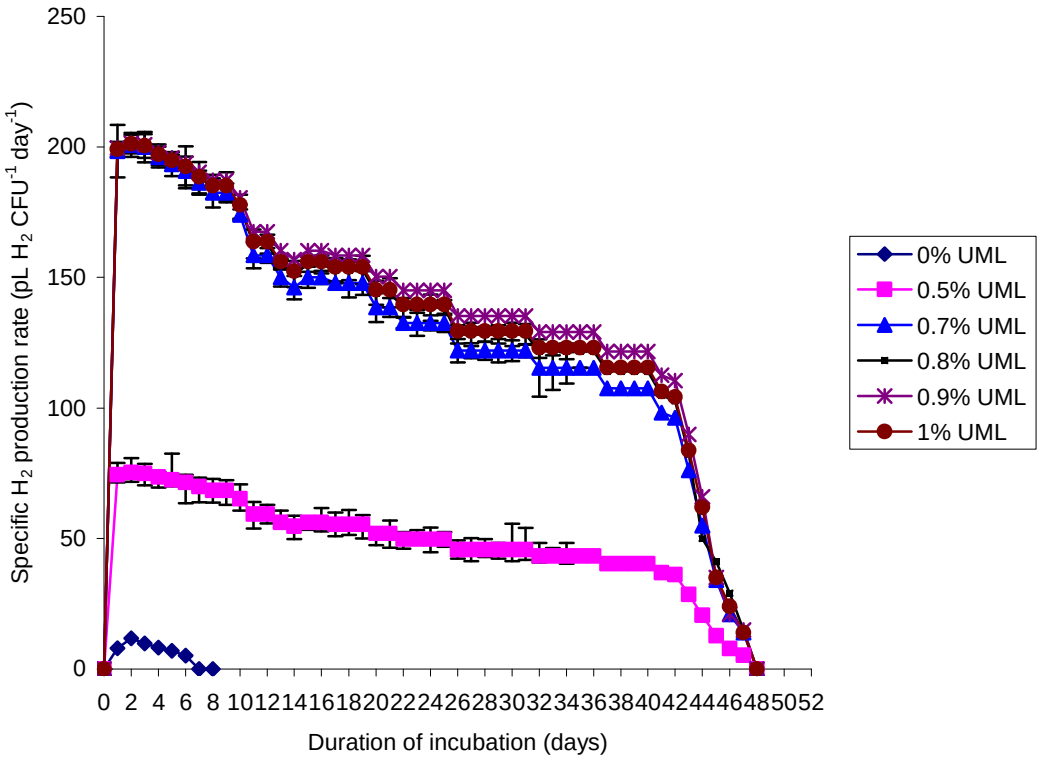


Figure 21. Daily hydrogen production by *C. saccharolyticus* grown on maize leaves as carbon source.

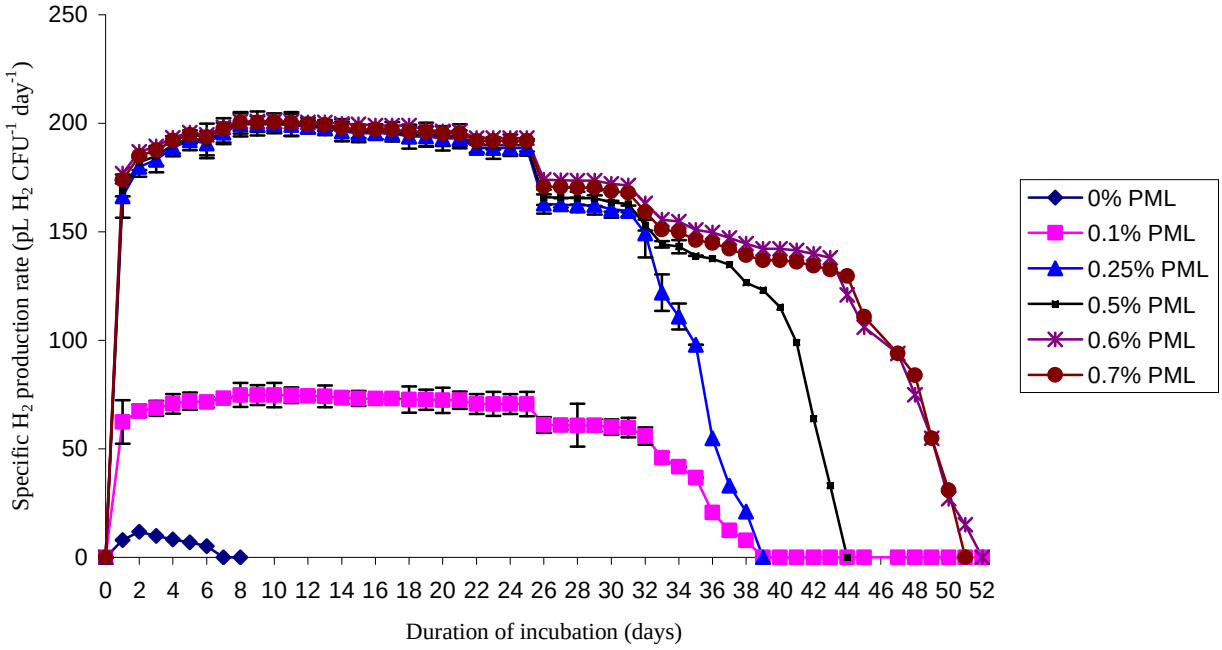


Figure 22. Daily hydrogen production by *C. saccharolyticus* grown on pretreated maize leaves as carbon source.

4.2.4. Sugarcane bagasse and silphium as carbon source for H_2 production

Sugarcane bagasse was also shown to be utilized by *C. saccharolyticus* for H_2 production. Sugarcane bagasse and sweet sorghum juice in 1% (w/v) concentrations respectively had the highest H_2 production rate (Fig. 19 and 23, Table 10 and 11) and showed a similar H_2 rate for a similar duration of fermentation – 34 and 38 days with about 30% and 50% drop of H_2 production rate in about 20-25 days, respectively.

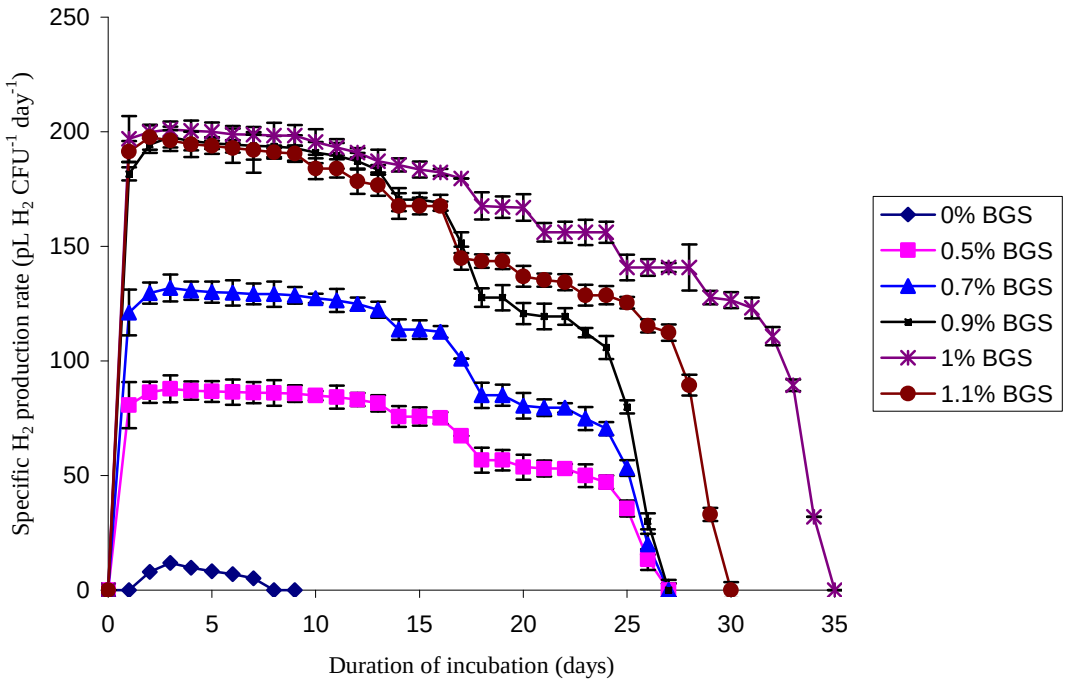


Figure 23. Daily hydrogen production by *C. saccharolyticus* grown on bagasse as a carbon source.

Sugarcane bagasse is as a byproduct of ethanol production in Latin America. Bagasse is either burnt, or used for biogas production. Some reports on hydrogen production from bagasse through steam gasification are available (Bowen et al. 2003; Chaudhari et al. 2003) and only few on biohydrogen production (Pattra et al. 2008). Optimisation of biohydrogen production from bagasse is at an early stage. In our study sugarcane bagasse was successfully utilized by *C. saccharolyticus* and had a H_2 rate similar to silphium (Table 11). BGS had a higher H_2 yield of 2.3 mol H_2/mol glucose consumed compared to the yield, reported by

Pattra et al. 2008 for *Clostridium butyricum* grown on sugarcane bagasse (1.73 mol H_2/mol total sugar).

Silphium (*Silphium trifoliatum*) as an energy plant has not been used so far for H_2 production. The addition of 1% silphium led to the highest H_2 production which lasted 38 days. (Fig. 24, Table 10). Thus we have shown that the extremophile *C. saccharolyticus* was capable to degrade silphium, resulting in H_2 yields, similar to those of PWS (Table 11).

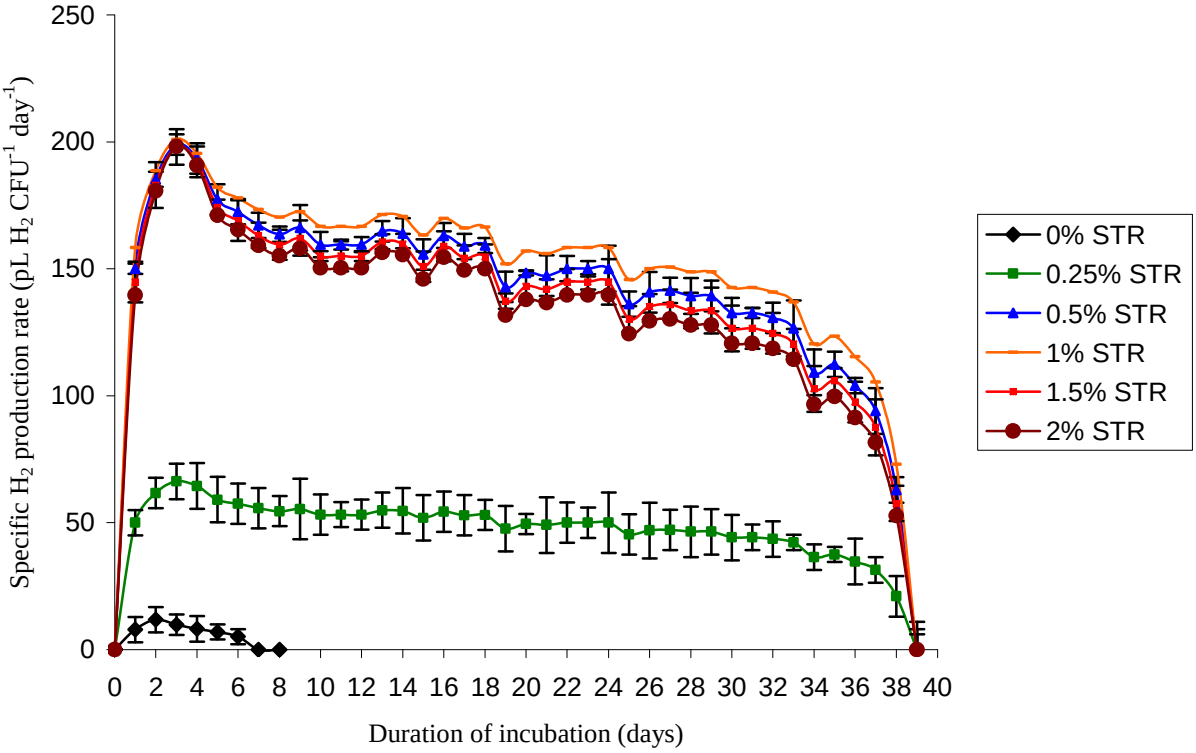


Figure 24. Daily hydrogen production by *C. saccharolyticus* grown on silphium as carbon source.

The contribution of silphium as a substrate for H_2 production by *C. saccharolyticus* was similar to that of sugarcane bagasse (Fig. 23, Table 10 and 11), thus possessing a potential to be used as a renewable energy source. Furthermore, it can be grown in territories with moderate climate, which makes it possible to be grown as an energy crop in Europe. Still, silphium was not as good substrate for H_2 production as bagasse, because the BGS productivity was 2.2-fold higher and the H_2 yield was about 5-fold higher than those of STR (Table 11).

4.2.5. Changes in TC, TOC and reducing sugars concentration

During the course of fermentation, the changes of the carbon content of the substrates was followed, both in the solid and liquid phase. The total carbon (organic and inorganic) of the solid compounds of three energy plants, i.e. wheat straw, sweet sorghum and pretreated maize leaves decreased in time with the highest decrement of about 24% over the first 15 days for wheat straw and 18% and 11% for sweet sorghum and pretreated maize leaves, respectively. After the 15th day of the fermentation only 5-6% decrease was observed for the three substrates (Fig. 25).

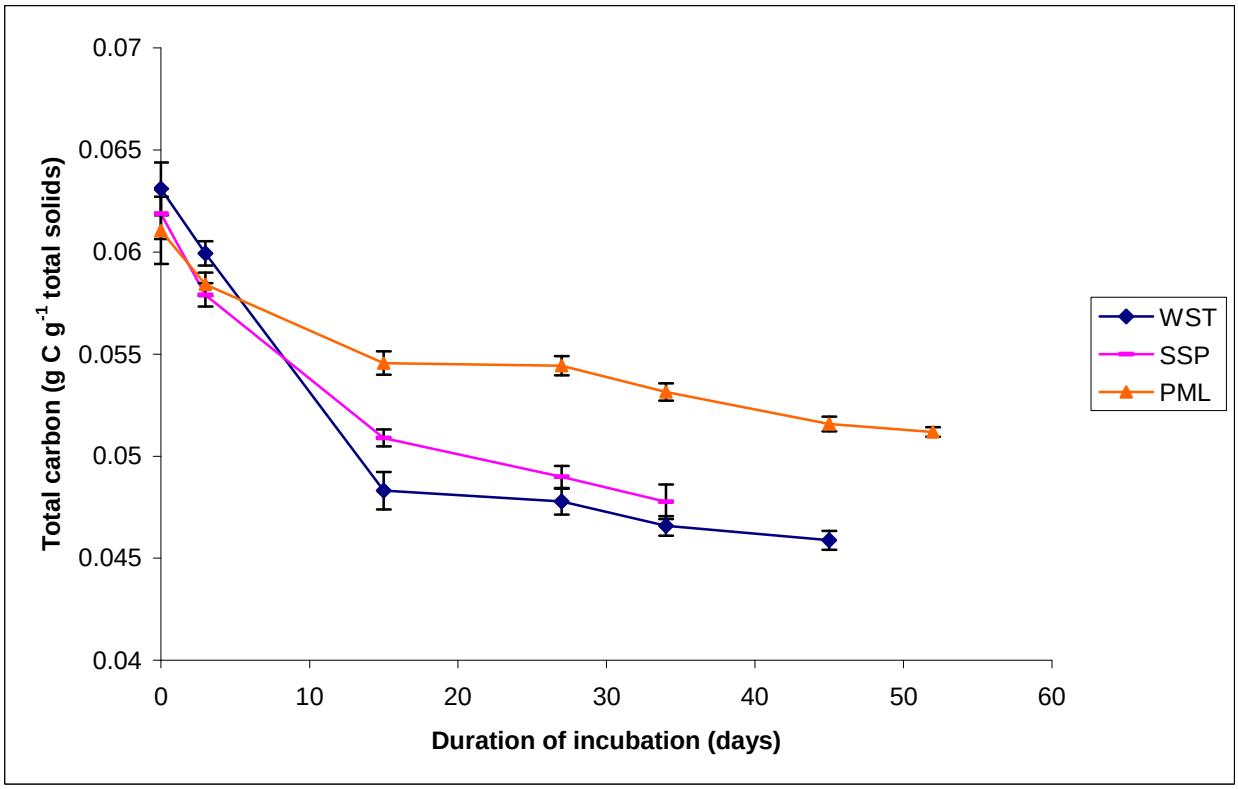


Figure 25. Total carbon of total solids.

The changes in the solubilised carbon of all used substrates have also been analyzed. There has been a gradual increase in the total organic carbon for all solid substrates and only a very little one for sweet sorghum juice and sweet sorghum concentrate 65 °Bx (Fig. 26). The highest increment for wheat straw, bagasse and pretreated maize leaves has been reached 27

days after the onset of the fermentation. For sweet sorghum that occurred by the 15th day. After that a slow decrease followed due to decomposition processes.

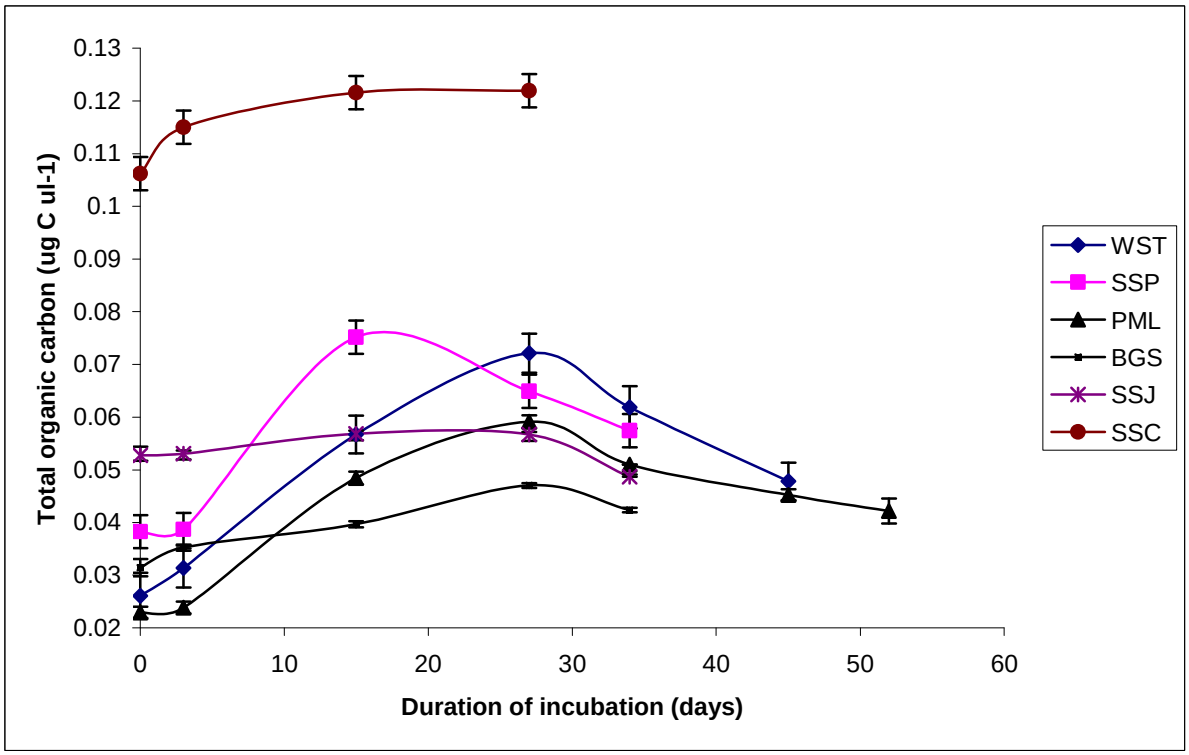


Figure 26. Changes of total soluble organic carbon released in time by *C. saccharolyticus* grown on biomass.

The concentration of the reducing sugars in the medium with and without cells has also been analyzed. The concentration of water-extractable sugars of the biomass in medium without cells was between 13 % and 20 % (data not shown). Hydrolysis of the lignocellulosic material by the enzymatic apparatus of *C. saccharolyticus* presumably took place after, or together with the consumption of the readily extractable sugars, causing accumulation of sugars in the extracellular fraction on the second and third day of fermentation, with a gradual decrease over time, due to their consumption by *C. saccharolyticus* (Fig. 27). The two sorghum juices had a higher initial concentration of reducing sugars relative to that of the solid biomass substrates, and were readily consumed by the bacterial cells, causing a decrease in the free sugar concentration in time (Fig. 27).

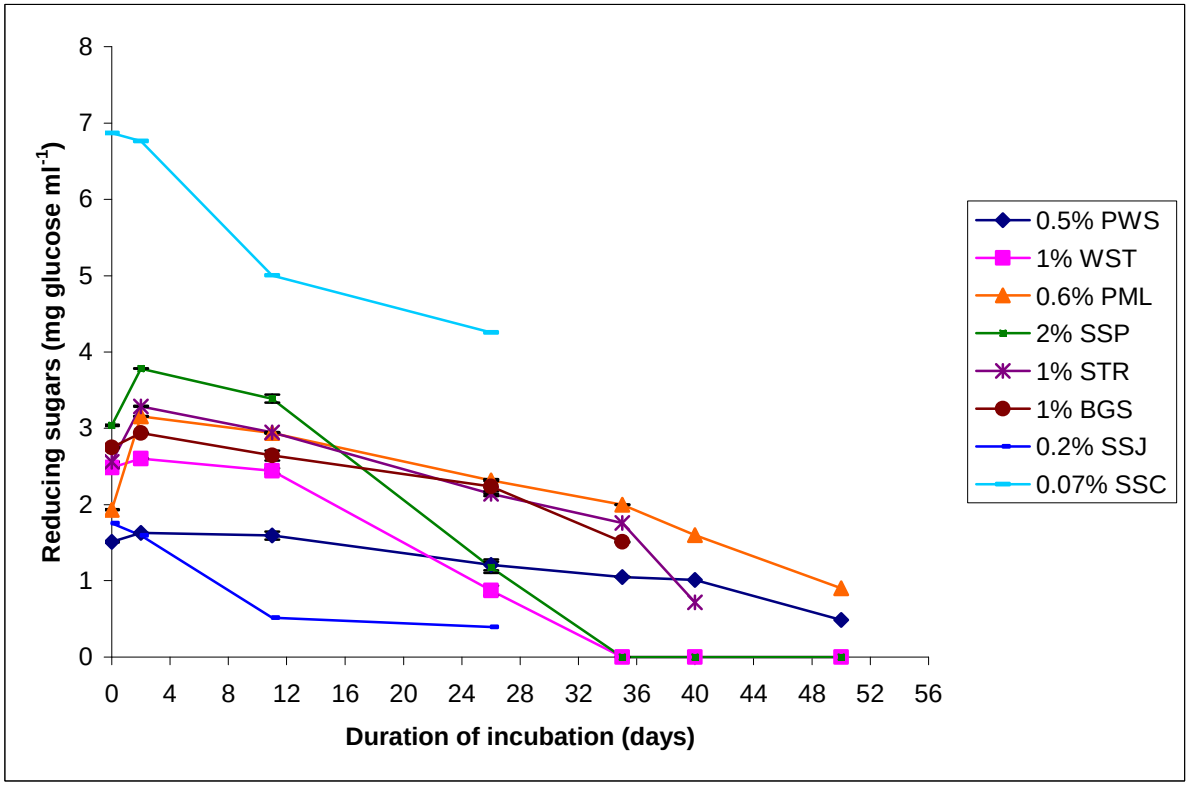


Figure 27. Glucose concentration in the extracellular fraction during the course of fermentation.

In conclusion, we have shown that *C. saccharolyticus* is able to degrade waste biomass materials of plant origin, producing hydrogen. Our data on H_2 production from wheat straw, sweet sorghum plants, pretreated maize leaves and sugarcane bagasse are comparable to the data published in the literature so far. In addition, we report for the first time on H_2 production from silphium. The results demonstrate that *C. saccharolyticus* is an excellent and reliable biocatalyst indeed with direct practical application in the biohydrogen-production technologies.

5. Summary

Based on the data, presented in this thesis, the following main conclusion can be drawn:

1. I demonstrated that *C. saccharolyticus* possesses an agarolytic activity in the presence of cellobiose, with a possible practical application in biohydrogen production, involving inexpensive and simple way of utilizing agarose as a discarded waste.

2. I performed preliminary studies on the protein profile of agarose-induced *C. saccharolyticus* cells, cultured in the presence of cellobiose and detected a number of newly synthesized proteins due to the use of agarose as an additional carbon and energy source.

3. I proved that *C. saccharolyticus* has the ability to catabolise alginic acid and alginates in the presence of cellobiose, and produces hydrogen from this substrate.

4. I confirmed the cellulolytic activity of *C. saccharolyticus* using pine tree wood shavings, demonstrating the metabolic versatility of that extremophile.

5. I have selected cheap, widely available and non-toxic support matrices for the stabilization of the hydrogen-producing system by immobilization.

6. I selected for a support matrix, which has a duel function for the cells – on one hand, providing a solid surface for the cells, and on the other, playing the role of a substrate, which maintains the viability and preserve the physiology status during storage.

7. The optimal storage conditions of *C. saccharolyticus* cells have been established and their storability has been improved by immobilization. The hydrogen productivity of immobilized cells has been preserved for a period of 30 days compared to 8 days of their freely suspended counterparts.

8. I demonstrated that energy plants and agricultural waste resources were suitable for use by *C. saccharolyticus* for hydrogen production, making that extremophile an excellent candidate for large scale operations of economically feasible biohydrogen generation.

6. Publications

Ivanova, G., Rakhely, G. and Kovacs, K. L. Hydrogen production from biopolymers by *Caldicellulosiruptor saccharolyticus* and stabilization of the system by immobilization. Int J Hydrogen Energy 2008; doi:10.1016/j.ijhydene.2008.08.058.

Ivanova, G., Rakhely, G. and Kovacs, K. L. Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus*. Submitted to Int J Hydrogen Energy.

Ivanova, G., Meredith, W., Dickinson, M. J., Kovacs, K. L. and West, H. M. Relationship between *n*-alkane length and fungal contribution to diesel degradation in an acidic sandy loam. Submitted to Bioresour Technol.

Ivanova, G., Meredith, W., Dickinson, M., Kovacs, K.L. and West, W. M. The contribution of species richness to diesel degradation. International Conference on Biotechnology, Leipzig, Germany, 9-13 July 2006.

Ivanova, G. Physiological studies on hydrogen-evolving and diesel-degrading microorganisms. Acta Biologica Szegediensis 2006;50(3-4):151.

Krysteva, M. Fyrtzov, K., Kodjabashev, I. and **Ivanova, G.** A substratum for hydrogen production. Patent utility model N: 681 / 03.09.2004, Patent Office of the Republic of Bulgaria.

Ivanova, G. Photobiological hydrogen production from vinasse by *Rhodobacter sphaeroides.* 4th International Conference on Biohydrogen, Ede, the Netherlands, 21-24 April 2002, pp.82.

Krysteva, M, Lalov, I. and **Ivanova, G**. Energy from food wastes - the energy of the 21st century (in Bulgarian). 4th National Chemistry Confernece, Sofia, Bulgaria, 27-29 September 2001, pp.153.

7. Aknowledgements

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I'm grateful to my Mother, who's been giving me all her love and comfort at difficult moments of my personal life and scientific career.

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8. Summary in Hungarian (Összefoglalás)

A dolgozatban bemutatott kísérleti eredmények alapján a következő következtetéseket vonom le:

1. Kimutattam, hogy *C. saccharolyticus* agar bontó enzim aktivitással rendelkezik cellobióz jelenlétében. A baktériumnak ez a tulajdonsága nem volt ismert korábban és lehetőséget nyújt arra, hogy agar tartalmú hulladékokból biohidrogént állítsunk elő.
2. A sejtekből készített fehérje extraktum gélképében eltéréseket találtam attól függően, hogy cellobióz mellett agar is jelen volt a tápfolyadékban vagy sem. Ez arra utal, hogy a polimer szubsztrát jelenlétében új fehérjék, enzimek indukálódnak, melyek pontos jellemzését újabb kísérletekkel kell elvégezni.
3. Kimutattam, hogy *C. saccharolyticus* képes lebontani cellobióz jelenlétében a güluronsavat, ami a természetben előforduló biopolimer alginát egyik komponense és bontja az alginátot magát is. Ezekből a polimerekből szintén hidrogént állít elő.
4. Megerősítettem azt a korábban leírt, de vitatott tényt, hogy a *C. saccharolyticus* sejtek cellulózbontó aktivitással rendelkeznek. A *C. saccharolyticus* sejtek hidrogéntermelés mellett hasznosították a puhafa forgácsban levő cellulózt és lignocellulózt termofil hőmérsékleten.
5. Olcsó hordozókon hatékony felületi immobilizálási eljárást dolgoztam ki a *C. saccharolyticus* sejtek számára.
6. Az immobilizálási körülmények optimalizálásával a sejtek életképességének jelentős növelését és biológiai aktivitásának hatékony megőrzését lehetett elérni a *C. saccharolyticus* számára kedvezőtlen életfeltételek mellett is.
7. Teszteltem különféle mezőgazdasági hulladékot és melléktermeket a *C. saccharolyticus* rendszerben és azt találtam, hogy a legtöbb alapanyag megfelelő szubsztrát a biohidrogén termelés számára. Ez és a *C. saccharolyticus* extrém termofil természete arra utal, hogy a vizsgált törzs kiválóan alkalmas ipari léptékű biohidrogén termelő rendszerekben való alkalmazásra.

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