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Biohydrogen Production in Extreme Conditions:
A Comprehensive Study of the Fermentative Metabolism of a Polyextremophilic Bacterium

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ABSTRACT

Dark fermentation is a potential carbon neutral process that exploits fermentative microorganisms to convert renewable organic substrates (e.g. lignocellulosic biomass and wastes) to H_2, a non-fuel commodity and an ideal and clean energy carrier for replacing fossil fuels in the future. In the quest for developing a robust and efficient dark fermentative process for H_2 production at industrial scale, the organism(s) selected to carry out the bioconversion is crucial. Thermophilic anaerobic bacteria have been drawing attention because they come close to meet the features that an ideal H_2-producing organism should possess, including efficient breakdown and conversion of complex organic substrates to H_2. In this study, a novel microorganism, *Caloramator celer* (former *Thermobrachium celere*), was evaluated for its potential to produce H_2 from organic substrates.

C. celer is a strict anaerobic, alkalitolerant, thermophilic bacterium capable of converting glucose to H_2, CO_2, acetate, ethanol and formate by mixed acid fermentation. In addition, *C. celer* shows remarkable features such as an extremely elevated growth rates (doubling time of 10 minutes) and the ability to grow in extreme conditions (T_{opt}= 67 °C; $pH^{67°C}_{opt}$= 8.2). For these reasons *C. celer* may be of industrial interest for the conversion of organic waste material to H_2 in an open (non-sterile) bioprocess system. However, for a biotechnological exploitation of this bacterium for H_2 production it is crucial to understand the factors that regulate carbon and electron flux and therefore the final distribution of metabolites to channel the metabolic flux towards the desired product.

The general goal of this study is to investigate the fermentative and energy metabolism of *C. celer* in order to understand how factors pertaining to the fermentation process can alter the metabolic fluxes. This is achieved by determining the relationship between fermentation conditions, physiological state, genome content, gene expression, metabolic fluxes and end-product yields through the combination of multiple methodologies such as conventional one-factor-at-a-time optimization, batch fermentations, comparative and functional genomics, transcription analysis and metabolic flux analysis. The final goal is to identify the optimal process conditions and metabolic state that maximize the H_2 production from *C. celer*.

In this study, glucose fermentation of *C. celer* was characterized in controlled and non-controlled cultivations and the effect of several parameters on growth and fermentation

of *C. celer* was investigated to identify the optimal conditions for H_2 production. In addition, the inhibitory effect of high concentrations of substrate and soluble end-products on growth and H_2 production was studied to assess the robustness of *C. celer*. The whole-genome sequence provided valuable information for interpretation of experimental results and for directing experimental design. Genomic data were employed to design transcriptional analysis, construct a stoichiometric model employed in metabolic flux analysis (MFA), and infer the metabolic network and possible regulatory mechanisms that dictate metabolic fluxes.

End-product synthesis profiles, and consequently H_2 production, changed in response to several modifications of the culture conditions namely growth rate, growth phase, iron content in the medium, substrate availability and nutrient content, presence of soluble metabolites, pH and H_2 concentration. The distribution of the fluxes at key metabolic nodes was found to be a function of thermodynamics as well as several physiological factors including genome content, growth and glycolytic rate, need for maintaining intracellular redox and pH homeostasis and only to some extent control of gene expression.

The synthesis of formate and ethanol, two products of the branched metabolism of *C. celer*, was found to compete with H_2-evolving reactions for the disposal of reducing equivalents. Ethanol and formate production served as an alternative to H_2 production for regulating the redox state when hydrogenases were inhibited. Moreover, formate synthesis was strictly linked to the growth rate suggesting its possible role in anabolic metabolism. Low growth rates, low substrate availability and nutrient content, high iron availability, presence of subinhibitory concentration of acetate and ethanol, slightly acidic pH and low H_2 concentrations minimized the redirection of carbon and electron flow to ethanol and formate synthesis and thus favored efficient H_2 production.

Kinetics of growth and H_2 production were inhibited, albeit to different degrees, by high concentration of substrate and soluble end-products, whereas H_2 yields remained marginally affected even in presence of considerable concentration of inhibitors. Acetate, the main soluble metabolite of the fermentation, inhibited H_2 productivity due to the increasing ionic strength in the medium, rather than the uncoupling effect of the undissociated form. The critical substrate and salt concentration estimated for *C. celer* suggests that this organism is not particularly osmotolerant.

In conclusion, this study provides valuable information on the capabilities of *C. celer* to efficiently produce H_2 as well as on its limitations through a comprehensive investigation of its fermentative and energy metabolism. *C. celer* showed a great metabolic flexibility that allows redistribution of fluxes at key metabolic nodes to simultaneously control redox state and efficiently harvest energy from substrate even under unfavorable conditions. Understanding how fermentation conditions control the

metabolic fluxes contributes to expand the knowledge of the thermophilic dark fermentative H_2 production process.

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LIST OF PUBLICATIONS

This thesis contains some unpublished material but is mainly based on the following original publications (I-VI), referred as roman numbers in the thesis.

I Ciranna A, Santala V, Karp M. 2011. Biohydrogen production in alkalithermophilic conditions: *Thermobrachium celere* as a case study. *Bioresource Technology* 102:8714-8722.

II Ciranna A, Santala V, Karp M. 2012. Enhancing biohydrogen production from the alkalithermophile *Thermobrachium celere*. *International Journal of Hydrogen Energy* 37:5550–5558.

III Ciranna A, Ferrari R, Santala V, Karp M. 2014. Effect of fermentative end-product inhibition on biohydrogen production by *Caloramator celer*: kinetic, metabolic and transcriptional analyses. *International Journal of Hydrogen Energy* 39:6391–6401.

IV Ciranna A, Pawar S, Santala V, Karp M, van Niel E. 2014. Assessment of metabolic flux distribution in the thermophilic hydrogen producer *Caloramator celer* as affected by external pH and hydrogen partial pressure. *Microbial Cell Factories* 13:48.

V Ciranna A*, Larjo A*, Kivistö A, Santala V, Roos C, Karp M. 2013. Draft genome sequence of the hydrogen- and ethanol-producing anaerobic alkalithermophilic bacterium *Caloramator celer*. *Genome Announcements* 1: e00471-13. *Equal contribution

VI Ciranna A, Kivistö A, Santala V, Karp M. 2014. A genomic insight into the carbon and energy metabolism of the alkalithermophile *Caloramator celer*. Manuscript.

THE AUTHOR´S CONTRIBUTION

I Alessandro Ciranna planned and performed the experimental work, and interpreted the results. He drafted the paper and is the corresponding author.

II Alessandro Ciranna planned and performed the experimental work, and interpreted the results. He drafted the paper and is the corresponding author.

III Alessandro Ciranna planned the experimental work and interpreted the results. Alessandro Ciranna and Roberto Ferrari performed the experiments. Alessandro Ciranna drafted the paper and is the corresponding author.

IV Alessandro Ciranna and Sudhanshu Pawar planned and performed the experimental work. Alessandro Ciranna interpreted the results, drafted the paper and is the corresponding author. Sudhanshu Pawar advised in the interpretation of the results.

V Alessandro Ciranna and Antti Larjo contributed equally to the publication. Alessandro Ciranna planned and performed wet-lab experiments, and contributed to data analysis. Antti Larjo planned and performed dry-lab experiments, and contributed to data analysis. Alessandro Ciranna drafted most of the paper and is the corresponding author. Antti Larjo helped in the writing of the manuscript. Anniina Kivistö advised in planning the experiments and interpreting the results. Christophe Roos advised in planning the experiments.

VI Alessandro Ciranna planned and performed the experimental work, and interpreted the results. He drafted the manuscript and is the corresponding author. Anniina Kivistö advised in planning the experiments.

The experimental work was carried out under the supervision of Prof. Matti Karp (Paper I-VI), Adjunct Prof. Ville Santala (Paper I-VI) and Associate Prof. Ed van Niel (Paper IV).

ABBREVIATIONS

(g)	Gas
(l)	Liquid
$[H_2]_{aq}$	Dissolved hydrogen concentration
$[S]_{crit}$	Critical substrate concentrations
$\Delta G^{0'}$	Standard Gibbs free energy
$\Delta G'$	Gibbs free energy
ABC	ATP-binding Cassette
Acetyl-CoA	Acetyl coenzyme A
ACK	Acetate kinase
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ALDH	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
bp	Base pair
C.	*Caloramator*
C_{50}	Half maximal inhibitory concentration
Ca.	*Caldicellulosiruptor*
Cal.	*Caldanaerobacter*
Can.	*Candida*
CDS	Protein-coding sequence
CDW	Cell dry weight
Ci.	*Citrobacter*
Cl.	*Clostridium*
COD	Chemical oxygen demand
CSTR	Continuous stirred-tank reactor
DNA	Deoxyribonucleic acid
E.	*Escherichia*
e^-	Electron
$E^{0'}$	Midpoint redox potential
ECH	Energy-conserving hydrogenase
ED	Entner-Doudoroff
EMP	Embden-Meyerhof-Parnas
En.	*Enterobacter*
Et.	*Ethanoligenens*
EU	European Union

F6P	Fructose 6-phosphate
FBA	Flux balance analysis
Fd_{ox}	Ferredoxin (oxidized form)
Fd_{red}	Ferredoxin (reduced form)
FeFe	Iron-iron
FeS	Iron-sulfur
FHL	Formate-hydrogen lyase
FNOR	Ferredoxin:$NAD(P)^+$ oxidoreductase
G+C	Guanine-cytosine
GAP	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAPOR	Glyceraldehyde-3-phosphate oxidoreductase
GHG	Greenhouse gas
Ha.	*Halanaerobium*
H_2ase	Hydrogenase
HS-CoA	Coenzyme A
HTR	Hydraulic retention time
K.	*Klebsiella*
kb	kilobase
K_c	Inhibition constant
MBH	Membrane-bound hydrogenase
MBX	Membrane-bound oxidoreductase
MEC	Microbial electrolysis cells
MFA	Metabolic flux analysis
MFC	Microbial fuel cell
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NFN	NADH-dependent ferredoxin:$NADP^+$ oxidoreductase
NiFe-	Nickel-iron
NMR	Nuclear magnetic resonance
P.	*Pyrococcus*
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PFL	Pyruvate-formate lyase
PFOR	Pyruvate:ferredoxin oxidoreductase
P_{H2}	Partial pressure of hydrogen
Pi	Orthophosphate
pK_a	Dissociation constant
PNS	Purple non-sulfur bacteria

PPDK	Pyruvate phosphate dikinase
PPi	Pyrophosphate
PPP	Pentose phosphate pathway
PTA	Phosphotransacetylase
PTS	Phosphotransferase system
R	Gas constant
rDNA	Ribosomal deoxyribonucleic acid
Rex	Redox-sensing protein
RM	Restriction modification
RNA	Ribonucleic acid
Rnf	*Rhodobacter* nitrogen fixation
rRNA	Ribosomal ribonucleic acid
T.	*Thermotoga*
T	Temperature
Tb.	*Thermobrachium*
TCDB	Transporter Classification Database
Thc.	*Thermococcus*
Th.	*Thermoanaerobacter*
The.	*Thermoanaerobacterium*
T_{opt}	Optimal temperature
tRNA	Transfer ribonucleic acid
YE	Yeast extract

1. INTRODUCTION

Energy is the main 'fuel' for social development and a foundation stone of the modern industrial economy. In 2011 the global total energy consumption was estimated at 590 EJ and in the worst case scenario this number is projected to increase by 61 % by 2050 (World Energy Council 2013). Therefore, since primary energy consumption will continue to rise, meeting both global and regional energy demand will be a challenge for the near future. Currently, the majority of the energy satisfying global demand is generated from fossil fuels (Figure 1). The dependency on fossil energy sources, however, contributes to environmental and societal problems such as the increase of CO_2 and greenhouse gas (GHG) emissions with consequent global climate changes, depletion of natural resources, geopolitical instability, fluctuation in oil price and supply, and public health risks (IPCC 2013). In order to simultaneously mitigate climate change and reduce the dependence on oil, alternative production chains are necessary.

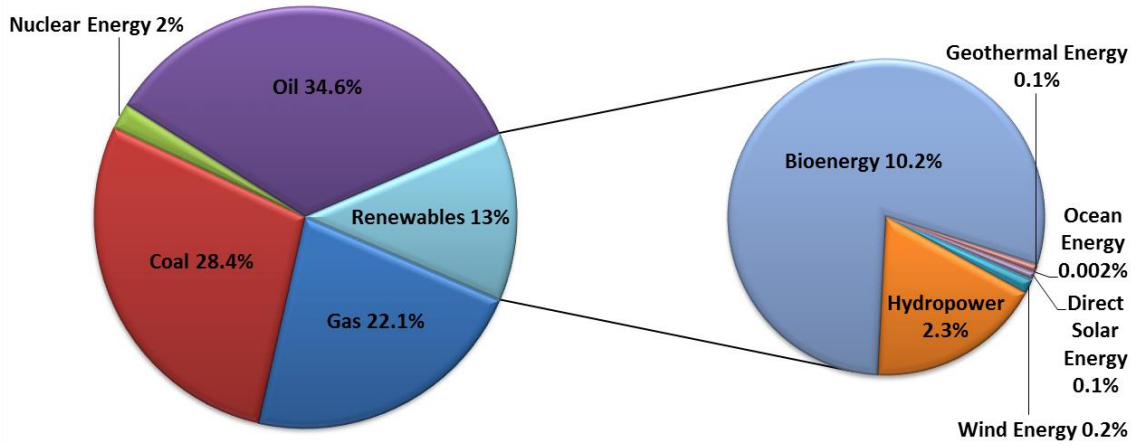

Figure 1. Shares of energy sources in total global primary energy supply in 2008 (modified from IPCC 2013, Special Report on Renewable Energy Sources and Climate Change Mitigation).

In the past two decades both public opinion and policy makers have grown awareness towards the lack of sustainability of the current world energy model. This has led to intensive research for alternative, clean and renewable sources to replace fossil fuels. Increasing the use of renewable energy to cover the global demand would contribute to decrease CO_2 and GHG emissions, to improve energy security, to promote rural development as well as to create new jobs (IPCC 2013). In 2008, 13 % of the total global primary energy supply derived from renewable sources including hydro,

wind, solar, geothermal, biomass and marine energy (IPCC 2013). Several countries are discussing and proposing ambitious targets to accelerate the further increment of this share in the near future (European Commission 2009, US Congress 2007, Hu et al. 2005). For example, the 2009 European Directive on the Promotion of Renewable Energy (European Commission 2009) sets the objective of reaching at least 20 % of the EU's energy consumption and 10 % of final energy consumption in the transport sector through renewable energy sources by 2020.

It is increasingly acknowledged globally that biomass has the potential to replace a large fraction of fossil resources as feedstocks for industrial productions, addressing both the energy and non-energy (e.g. chemicals and materials) sectors (European Commission 2004). In 2008, energy from biomass represented 10.2 % of the annual global energy, resulting to be the largest contributor to energy from renewable sources. A variety of processes allows biomass feedstocks to be used directly to generate electricity or heat, or to produce biofuels in gaseous, liquid, or solid form (Srirangan et al. 2012). Although a bioenergy-based economy can potentially contribute to decrease CO_2 and GHG emissions and improve energy security, the sustainability of bioenergy generation heavily depends on the nature of the feedstocks employed in the process and how they are grown, converted, and used (Solomon 2010). 1st generation biofuels produced primarily from food crops (e.g. corn, sugarcane, rapeseed) have been under scrutiny mainly for competing with agriculture for arable land and consequently negatively affecting food prices and supplies. On the other hand, 2nd generation biofuels produced from non-food biomass (e.g. forest, agricultural and livestock residues, short-rotation forest plantations, energy crops, organic component of municipal solid waste and other organic waste streams) do not directly compete with food production allowing better land use opportunities (Sims et al. 2009). Finally, feedstocks for production of 3rd generation biofuels (i.e. microalgae and seaweeds) are produced on non-agricultural land or marine conditions overcoming the disadvantages of 1st and 2nd generation biofuels (Li et al. 2008). To correctly assess and compare the sustainability of different biofuel production processes the environmental, social and economic impact should be thoroughly analyzed by covering the entire life cycle of a product: raw material, production, manufacture, product use and fate (Hatti-Kaul et al. 2007).

Several energy carriers can be produced from biomass (e.g. biodiesel, ethanol, butanol, syngas, methane and hydrogen). All of them present advantages as well as disadvantages in the ambitious aim of replacing fossil fuels (Srirangan et al. 2012). Hydrogen (H_2) is considered by many as one of the most promising alternatives to replace fossil-based energy carriers (Schrope 2001, Balat 2008, Veziroglu and Şahin 2008). Hydrogen is the simplest and most abundant element in the universe. However, H_2 does not occur naturally as a gas on Earth, being always combined with other elements (Momirlan and Veziroglu 2005). A primordial concept of hydrogen economy

was already envisioned in 1874 by Jules Verne in his novel *Mysterious Island* where, based on the awareness of finite supply of coal and the possibilities of producing hydrogen by water electrolysis (Turner 2004), he wrote:

> *"Yes my friends, I believe that water will one day be employed as fuel, that hydrogen and oxygen which constitute it, used singly or together, will furnish an inexhaustible source of heat and light, of an intensity of which coal is not capable....When the deposits of coal are exhausted we shall heat and warm ourselves with water. Water will be the coal of the future" (Verne 1874)*

Currently, hydrogen economy is defined as the utilization of hydrogen to transport energy from renewables over large distances; and to store it (for supply to cities) in large amounts (Bockris 2002). Indeed, as an energy carrier, H_2 possesses many desirable properties such as high energy content (122 kJ/g), clean combustion, high efficiency conversion to usable energy (heat or electricity) and storability (Busby 2005, Balat 2008, Balat and Kırtay 2010). In a H_2-based economy this compound can be used as vehicle fuel, for stationary production of electricity and heat, and as a fuel for portable electronics (Table 1). However, at present its commercial use as fuel or energy carrier is limited to few applications, for example as a rocket fuel and electricity source in spacecrafts (Ramachandran and Menon 1998, Das and Veziroğlu 2001, Busby 2005). Nevertheless, despite the several barriers that hinder the employment of H_2 as an alternative source of energy (i.e. the immaturity of the technology, the lack of commercial competitive technology and infrastructures, and economical risks) (Busby 2005, Marbán and Valdés-Solís 2007, van Ruijven et al. 2007, Winter 2009), several economic and technological efforts are presently underway to find solutions to make hydrogen energy a commercially viable alternative to fossil fuel and examples of application of hydrogen technology in the transportation sector (e.g. H_2 fuel cell vehicles and H_2 filling stations) are already available (European Hydrogen Association 2014). Today, H_2 is mainly used as a non-fuel commodity in a variety of industrial chemo-physical processes (Table 1), especially in oil refinery that in 2008 accounted for nearly 90 % of the global H_2 consumption (Ramachandran and Menon 1998, Busby 2005, Lattin and Utgikar 2007, Freedonia 2010).

Table 1. Main applications of hydrogen (Ramachandran and Menon 1998, Busby 2005, Winter 2009)

Application	**Function**
Petrolchemical industry	Cracking and hydrogenation of hydrocarbons
	Upgrading heavy crude oils into refined fuels to meet increasingly tight transportation fuel specifications
Fertilizer industry	Production of ammonia
Chemical industry	Production of chemicals (methanol, acetic acid, butanediol, tetrahydrofuran, hexamethylene, cyclohexane, polypropylene, etc.)
Food industry	Oil and fat hydrogenation
Metallurgical application	O_2 scavenger
	Reductant for recovery of nickel, cobalt and copper
Mechanical industry	Enhancing plasma welding and cutting operations
	Mixed with argon for welding stainless steel
Electricity supply	Cooling of generators, motors and transformers Gas turbine/steam turbine combined cycles
Electronics industry	Production of silicon wafer and circuit under reduced conditions
Nuclear industry	O_2 scavenger to prevent corrosion
Glass manufacture	O_2 scavenger in float glass manufacture
	Glass cutting with H_2 flame
Weather monitoring	H_2-filled weather baloons
Space exploration	Rocket fuel
	Powering life-support systems and computers in space environments
Transportation sector[a]	Fuel cell vehicles Hydrogen-fueled internal combustion engine
Commercial sector[a]	Portable and stationary fuel cell for power and heat applications
Household[a]	Portable and stationary fuel cell for power and heat applications

a) Key technical barriers still present for a state-of-the-art technology

The global demand for hydrogen in 2013 was forecast to be 475 billion m^3 (Freedonia 2010). Presently, about 95 % of global H_2 production is based on fossil fuels being steam reformation of natural gas (48 %), thermochemical gasification of coal (18 %), naphtha and heavy oils (30 %) the most employed processes. Only 4 % and 1 % is generated from water using electricity and biomass, respectively (Das and Veziroglu 2008, Balat and Kırtay 2010). These methods are all energy intensive processes and not carbon neutral (Turner 2004). As a carrier and not an energy source, H_2 can only be as clean as the method employed for its production. In order to develop a sustainable hydrogen economy, hydrogen production technologies need to utilize renewable sources.

Several biological processes can be exploited to convert renewable feedstocks to molecular H_2. Indeed, a variety of microorganisms belonging to all three domains of life (archaea, prokaryotes and eukaryotes) are capable of synthetizing molecular H_2 during their metabolism. The first observations of H_2 production from microalgae and bacteria dated back to late 1800s. However, basic research in this field started decades later with the study of bacterial (late 1920s) and microalgal H_2 production (1940s). Only in the early 1970s applied research and development on photobiological H_2 production began, while at first dark fermentation was relatively neglected (Hallenbeck and Benemann 2002). The basic function behind the production of H_2 in these microorganisms is to dispose reducing equivalents by reactions catalyzed by H_2-producing enzymes (hydrogenase or nitrogenase). These metalloproteins containing iron-sulfur (FeS) clusters are capable of reducing solvent protons to H_2. Nitrogenases catalyze the

irreversible reduction of nitrogen to ammonia using low potential reductants (ferredoxin or flavodoxin) as electron donors (Jackson et al. 1968, Tamagnini et al. 2002, Vignais et al. 2006) according to the reaction:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (1)$$

However, in absence of N_2 the total electron flux is directed to the protons (Vignais et al. 2006). The simplest chemical reaction occurring in nature, the reversible reduction of protons, is catalyzed by hydrogenases (H_2ases) (Vignais and Billoud 2007, for more details see section 3.1.2):

$$2H^+ + 2e^- \leftrightarrow 2H_2 \quad (2)$$

Biological H_2 production processes include photolysis, dark- and photofermentation, water-gas shift reaction and electrochemically assisted microbial fuel cell. A summary and comparison of principles, reactions, microbiology, advantages and disadvantages of different biological H_2 production methods is presented in Table 2. These methods are more environmentally friendly and less energy intensive than thermochemical and electrochemical processes (Das and Veziroğlu 2001). In addition, when produced from renewable resources, biohydrogen (H_2 produced by microorganisms) is a carbon-neutral energy carrier or even potentially carbon-negative if the CO_2 released during the process is captured and stored (Hallenbeck and Ghosh 2012). Currently, biological H_2 production is however still at a "Research & Development" stage since several technical and economical limitations hinder its efficient transition to commercial scale (Das and Veziroglu 2008, Kotay and Das 2008).

The present thesis focuses on the characterization of a novel bacterium for the generation of H_2 by dark fermentation at high temperatures. In the literature survey the background information about principles, microbiology, thermodynamics and challenges of dark fermentative biohydrogen production is presented with emphasis on fermentative thermophilic microorganisms. In the experimental part of this study a novel anaerobic, thermophilic and alkalitolerant bacterium, *Caloramator celer*, is presented for its potential to produce H_2 from organic substrates. The study investigates the chemo-physical and nutritional factors that influence the performance of this organism in pure culture. In addition, the fermentative metabolism of *Caloramator celer* was explored by combining physiological, genomic and computational approaches to characterize potential bottlenecks limiting H_2 production, and to identify the suitable conditions to develop an efficient H_2 production process using this thermophilic bacterium.

Table 2. Summary of biological processes for hydrogen production (modified from Koskinen 2008a).

Mechanism	Reaction	Organisms	Enzyme(s)	Advantages	Disadvantages	References
Direct Photolysis Photosystem II and I capture solar energy to split water producing O_2 and Fd_{red}. A hydrogenase or nitrogenase reoxidized Fd_{red} producing H_2.	$2H_2O \rightarrow 2H_2 + O_2$	Photoautotrophic microorganisms (green microalgae, cyanobacteria)	Hydrogenase (microalgae, cyanobacteria) Nitrogenase (cyanobacteria)	+ H_2 production from water + No need for organic electron donors (other than CO_2) + Higher solar light conversion efficiency than plants	- Discontinuous process - Low production rates - End-product inhibition by O_2 - Fire hazard from O_2 in the product	for reviews, see Benemann 1996, 1997; Das and Veziroglu 2001; Levin et al. 2004; Kapdan and Kargi 2006; Kovacs et al. 2006; Redwood et al. 2009
Indirect Photolysis CO_2 is fixed to synthesize simple sugars. Stored carbohydrates can be metabolized fermentatively to generate H_2 indirectly.	$6H_2O \rightarrow C_6H_{12}O_6 + 9O_2$ $C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$					
Photofermentation H_2 catalyzed in anaerobic conditions by nitrogenase under N_2-deficient conditions using light energy and reduced compounds (organic acids).	$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$	Photoheterotrophic bacteria (purple non-sulfur bacteria)	Nitrogenase	+ H_2 production from various carbohydrates and organic wastes + High H_2 yields + Relatively high production rates + Oxidation of organic acids	- CO_2 present in the product gas - Effluent treatment required - Low light energy conversion - Impurity of product gas (e.g. H_2S) -Requires light, discontinuous if not illuminated - PNS nitrogenases not compatible with NH_4^+	for reviews, see Das and Veziroglu 2001; Hallenbeck and Benemann 2002; Levin et al. 2004; Kapdan and Kargi 2006
Dark fermentation In anaerobic conditions, oxidation of organic substrates produces reducing equivalents in form of NADH, Fd_{red} and formate. Oxidation of these molecules by hydrogenases generates H_2.	$C_6H_{12}O_6 + 6H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$ $C_6H_{12}O_6 + 6H_2O \rightarrow CH_3CH_2COOH + 2H_2 + 2CO_2$	Heterotrophic microorganisms (anaerobic archaea and bacteria)	Hydrogenase	+ H_2 production from various carbohydrates and organic wastes + High H_2 production rates + No light required + Simpler process + Simultaneous production of value-added compounds (e.g. ethanol, 1,3-propanediol)	- CO_2 present in the product gas - Incomplete oxidation of organic material - Low H_2 yields -Generally low conversion efficiencies of substrates - Effluent treatment required - Impurity of product gas (e.g. H_2S) - Unstable continuous H_2 production - Hydrogenase sensitive to P_{H2}	for reviews, see Das and Veziroglu 2001; Hallenbeck and Benemann 2002; Levin et al. 2004; Kapdan and Kargi 2006; Kotay and Das 2008; Das and Veziroglu 2008; Hallenbeck 2009; Redwood et al. 2009; Wang and Wan 2009
Water-shift reaction CO-dehydrogenase and hydrogenase catalyzed the oxidation of CO to CO_2 with the release of H_2.	$CO(g) + H_2O(l) \rightarrow H_2(g) + CO_2(g)$	Photoheterotrophic bacteria (purple non-sulfur bacteria) Heterotrophic microorganisms (anaerobic archaea and bacteria) Chemolithotrophic bacteria	Carbon monoxide dehydrogenase Hydrogenase	+ CO removal (e.g. from gas streams) + Relatively high production rates + No light required	- CO_2 present in the product gas - Low mass transfer	for reviews, see Benemann 1996, 1997; Levin et al. 2004
Electrochemically assisted microbial fuel cell By supplying a small input of electrical energy to a MFC, the protons formed during oxidation of organic sources at the biological anode are reduced to H_2 at the cathode.	$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2$	Electrochemically active bacteria	Cytochromes Microbial nanowires	+ Oxidation of organic acids + High H_2 yield	- CO_2 present in the product gas - Low efficiency - Low H_2 production rates	for reviews, see Angenent et al., 2004; Logan 2004; Hawkes et al., 2007; Cheng and Logan 2007; Lovley 2008

1.1. DARK FERMENTATION AND HYDROGEN PRODUCTION

Molecular H_2 is one of the final products of the light-independent anaerobic degradation of organic substrates by heterotrophic organisms, also known as dark fermentation. In nature this biological process is an intermediate stage in the anaerobic digestion of organic matter (Pavlostathis and Giraldo-Gomez 1991, Stams 1994). When the methanogenic reactions are inhibited by suppressing growth of H_2-consuming microorganisms or by culturing axenic cultures (pure cultures containing clonal microbial populations) of fermentative microorganisms, H_2 can be produced through dark fermentation (Pavlostathis and Giraldo-Gomez 1991). Since fermentative microorganisms lack the ability to use terminal electron acceptors, the reduction of protons to H_2 serves as one of the possible ways to dispose the excess of reducing power generated in the oxidation of organic compounds (Stams 1994). Dark fermentation is generally recognized to have several advantages such as being light-independent, wide substrate versatility, high H_2 production rates, low energy demand and simplicity of the process (Hallenbeck and Benemann 2002, Kotay and Das 2008, Das and Veziroglu 2008, Wang and Wan 2009). Therefore, fermentative microorganisms (strict and facultative anaerobes) can combine H_2 production with degradation of a variety of renewable feedstocks and waste materials making the process potentially sustainable. However, this bioprocess is not mature yet and major challenges (e.g. low H_2 conversion efficiency, H_2 separation and purification, stability of the process, design of full-scale application) need to be addressed to compete with commercial H_2 production processes from fossil fuels in terms of cost, efficiency and reliability (Das and Veziroglu 2008, Show et al. 2012).

1.1.1. Biochemical principles of fermentative H_2 production

The complete oxidation of one molecule of glucose yields 12 molecules of H_2 according to the reaction:

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6HCO_3^- + 6H^+ \quad (3)$$

However, in fermentative H_2-producing microorganisms the oxidation of carbohydrates is generally carried out by mixed-acid fermentation resulting in the synthesis of several metabolites (Table 3). The generation of reduced metabolites (e.g. H_2, lactate, ethanol, alanine, acetone, butyrate, butanol, succinate and propionate) is a means for fermentative microorganisms to regenerate oxidized electron carriers (NAD^+ and/or Fd_{ox}) allowing to sustain their catabolic metabolism (Stams 1994). Consequently, most of the electrons remain bound to other by-products lowering the overall H_2 yield of dark fermentation (Table 3).

Table 3. Products, cofactors, intermediates and ATP generated by reactions in anaerobic fermentation (modified from Temudo et al. 2007).

Product	$Y_{prod/glu}$	$Y_{NADH/glu}$	$Y_{Fdred/glu}$	$Y_{AcCoA/glu}$	$Y_{ATP/glu}$	$Y_{H2/glu}$	$\Delta G^{0'}$ (kJ/mol)[a]
Acetate	2	2	2	2	4	4	-206.3
Ethanol	2	-2	2	2	2	0	-235.0
Butyrate	1	0	2	2	3	2	-154.8
Butanol	1	-2	2	2	2	0	-280.5
Lactate	2	0	0	0	2	0	-198.1
Propionate	2	-2	0	0	2	0	-359.0
Succinate	2	-2	0	0	2	0	-325.5
Alanine	2	0	0	0	2	0	-174.1

a) calculate based on the Gibbs free energies of formation provided by Thauer et al. 1977

Generally, most of the fermentative H_2 producers (i.e. enteric bacteria and clostridia) utilize the Embden-Meyerhof-Parnas (EMP) pathway to generate pyruvate from hexose (Hallenbeck and Ghosh 2012) yielding 2 ATP and 2 NADH per molecule of glucose metabolized (Figure 2). Pyruvate, a key intermediate of the fermentation, can be catabolized by two different metabolic routes which in turn affect how H_2 is produced (Hallenbeck and Benemann 2002, Hallenbeck 2009, Hallenbeck and Ghosh 2012). Both facultative (i.e. enteric bacteria) and strict (i.e. clostridia and *Thermococcales*) anaerobes convert pyruvate to acetyl-CoA, but generally the former utilize pyruvate-formate lyase (PFL) with simultaneous production of formate, while the latter utilize pyruvate:ferredoxin oxidoreductase (PFOR) with simultaneous production of CO_2 and reduced ferredoxin (Fd_{red}) (Figure 2).

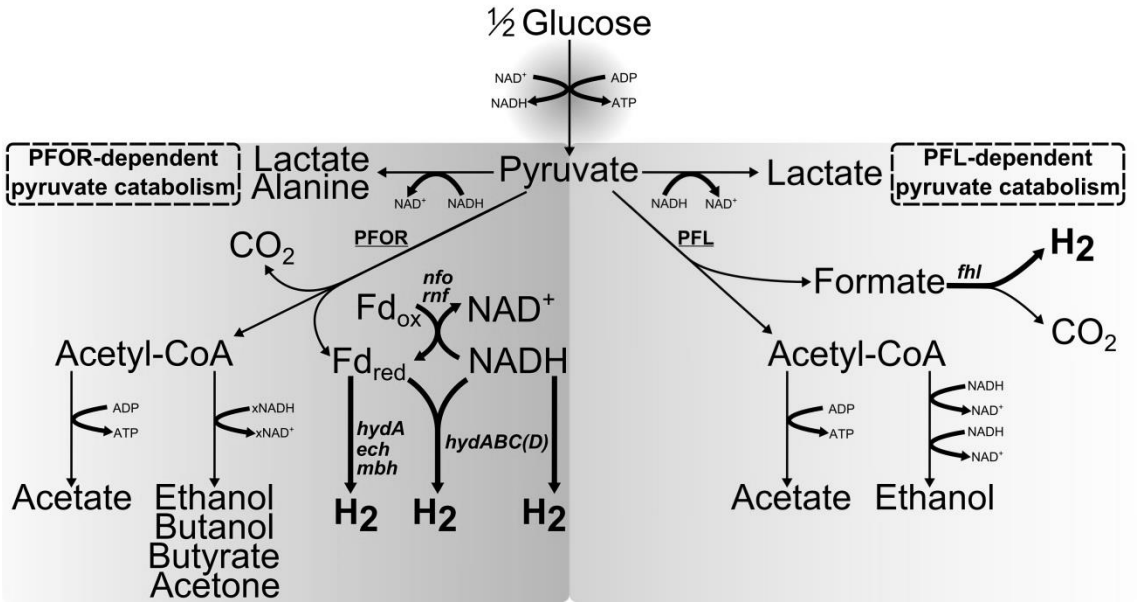

Figure 2. General metabolic pathways in dark fermentative H_2 production (modified from Hallenbeck and Ghosh 2012).

In facultative anaerobes H_2 is derived from formate by formate-hydrogen lyase (FHL) complex, a membrane-bound NiFe-H_2ase (Hallenbeck and Benemann 2002, Hallenbeck 2009, Hallenbeck and Ghosh 2012) (Figure 2). In this microbial group, the excess of

reducing power (NADH) is generally disposed by conversion of acetyl-CoA to ethanol or by reduction of pyruvate to lactate. The incomplete degradation of formate to H_2, the inability of utilizing the reducing power of NADH for H_2 production and the synthesis of other reduced metabolites limit the H_2 yield of enteric bacteria to a maximum of 2 mol H_2/mol glucose (Hallenbeck and Benemann 2002, Hallenbeck 2009, Hallenbeck and Ghosh 2012).

In clostridia-like microorganisms all the reducing equivalents produced during carbohydrate catabolism (2 NADH and 4 Fd_{red} per glucose) can be theoretically disposed by H_2 production. The direct oxidation of Fd_{red} and NADH via proton reduction can be catalyzed by a variety of H_2ases using Fd_{red} (Adams et al. 1989, Sapra et al. 2003, Soboh et al. 2004), NADH (Soboh et al. 2004) or both (Schut and Adams 2009) as electron donors (Figure 2). Alternatively electrons can be transferred between cofactors by ferredoxin:NAD^+ oxidoreductases (Biegel et al. 2011). In strictly anaerobic H_2 producers a theoretical maximum yield of 4 mol H_2/mol glucose can be achieved when all the reducing equivalents are transferred to H_2, and acetate and CO_2 are the only other metabolic products of the fermentation (Eq. 4) (Thauer et al. 1977).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2HCO_3^- + 2CH_3COO^- + 4H^+ \quad (4)$$

However, H_2 production is thermodynamically constrained (Stams 1994, Verhaart et al. 2010) and when reductant disposal through proton reduction is inhibited the accumulated reducing equivalents in the cell are recycled by reduction of pyruvate or acetyl-CoA to other metabolites such as lactate, ethanol, acetone, butyrate, butanol or alanine (Verhaart et al. 2010). Both H_2 and ATP yields decrease as a consequence of the changes in the fermentation profile (Table 3).

1.1.2. Organisms for fermentative H_2 production

A wide variety of microorganisms with vastly different taxonomic and physiological characteristics can produce H_2 by fermentative metabolism. These include facultative or strictly anaerobic bacteria, archaea and yeasts. Thus far, the most studied microbial groups for dark fermentative H_2 production are the family of *Clostridiaceae* and *Enterobacteriaceae*, although strains belonging to *Thermoanaerobacterales* (Family III), *Thermoanaerobacteraceae*, *Thermotogales* and *Thermoccoccales* have received scientific attention (Rittmann and Herwig 2012). The ideal H_2 producer should: i) generate H_2 at high yields and productivities, ii) degrade a wide range of biomass, iii) metabolize both hexose and pentose simultaneously, iv) tolerate high concentrations of sugars and fermentation products, v) withstand growth inhibitors from biomass feedstock or waste materials, vi) grow in relatively inexpensive growth medium, vii) tolerate oxygen, viii) shift the metabolism to useful by-products when under stress, ix)

be genetically tractable (van Niel et al. 2011, Pawar and Van Niel 2013). Thus far, none of the investigated organisms completely fulfills all these criteria, although *Caldicellulosiruptor* spp. and *Thermotoga* spp. are recognized as the closest to the ideal H_2 producer (Pawar and Van Niel 2013). However, the concept of a single ideal microorganism suitable for developing a universal approach to establish an efficient, cost-effective and sustainable H_2 production process may not be realistic. Instead, the selection of the proper organism(s) for H_2 production will most likely depend on the process parameters (e.g. feedstocks and culture conditions).

Table 4. Examples of use of pure cultures, mixed cultures and designed co-cultures for fermentative H_2 production from model substrates.

Organism/ Source of inoculum	Substrate	Operational condition	H_2 Yield[a]	Q_{H2}[b]	Major soluble metabolites[c]	References
Pure cultures						
Cl. beijerinckii	Glucose	Batch^M	2.8	ND	Ace, but, butOH	Lin et al. 2007
Cl. butyricum	Glucose	Batch^M	2.3	ND	Ace, but, lac, pro, for	Lin et al. 2007
Cl. tyrobutyricum	Glucose	Batch^M	3.2	ND	Ace, but, etOH, lac	Jo et al. 2008
En. cloacae	Sucrose	Batch^M	3.0	35.6	ND	Kumar and Das 2002
E. coli W13	Glucose	Fed Batch^M	1.1	500	Ace, lac, suc, etOH, for, mal	Yoshida et al. 2006
Ha. saccharolyticus	Glycerol	Batch^M	2.2	ND	Ace, 13-pdiol	Kivistö et al. 2011
Ca. saccharolytuicus	Glucose	Continuous^T	4.0	12.4	Ace	De Vrije et al. 2007
C. celer	Glucose	Batch^T	3.5	42.0	Ace, etOH, form	Ciranna et al. 2012
T. maritima	Glucose	Batch^T	4.0	6.9	Ace	Schroder et al. 1994
P. furiosus	Maltose	Continuous^T	2.9	22.0	Ace, pro, but, bVFA	Schicho et al. 2006
Mixed cultures						
Hydrogenogenic labscale CSTR	Sucrose	Continuous^M	2.1	22.0	Ace, but, etOH, metOH, propOH, butOH, val, bVFA	Fang and Liu 2002
Wastewater treatment plant	Sucrose	Continuous^M	1.9	387	Ace, but, etOH, pro, val	Lee et al. 2006
Wastewater treatment plant	Sucrose	Continuous^M	1.6	628	Ace, but, etOH, pro	Wu et al. 2006
Geothermal spring, Hveravellir, Iceland	Glucose	Continuous^T	1.5	46.0	Ace, but, etOH, lac, for	Koskinen et al. 2008b
Hydrogenogenic labscale CSTR	Glucose	Continuous^T	2.5	2.1	EtOH, butOH, ace, pro, but, val, lac, for	Kotsopoulos et al. 2006
Slurry of cow manure	Glucose	Continuous^T	1.7	1.6	Ace, but lac, etOH	Yokoama et al. 2007
Designed co-cultures						
Cl. butyricum & *E. coli*	Glucose	Batch^M	1.7	ND	Ace, lac, but, etOH	Seppälä et al. 2011
En. aerogenes & *Can. maltosa*	Glucose	Batch^M	2.2	11.6	Ace, etOH, 23-bdiol, lac, but, suc	Lu et al. 2007a
Ca. saccharolyticus & *Ca. kristjanssonii*	Glucose/ xylose	Continuous^T	3.7	11.6	Ace, lac	Zeidan and van Niel 2011
Ca. saccharolyticus & *Ca. owensensis*	Glucose/ xylose	Batch^T	3.3	16.0	Ace, lac, etOH	Zeidan and van Niel 2009

ND, not detected; M, mesophilic condition; T, thermophilic condition

a) mol H_2/mole hexose equivalent

b) volumetric H_2 production rate (mmol H_2/l/h)

c) major soluble metabolites are abbreviated as follows: 13-pdiol, 1,3-propanediol; 23-bdiol, 2,3-butandiol; ace, acetate; but, butyrate; butOH, butanol; bVFA, branched volatile fatty acids; etOH, ethanol; for, formate; lac, lactate; mal, malate; metOH, methanol; pro, propionate; proOH, propanol; suc, succinate; val, valerate

Hydrogen production by dark fermentation has been investigated using pure cultures, mixed cultures or designed co-cultures as inoculum sources (Table 4). Pure cultures are mainly used in lab-scale reactors to study the H_2 production potential of newly isolated strains (O-Thong et al. 2008a, Zhao et al. 2011), physiological and biochemical properties of H_2-producing organisms (Sapra et al. 2003, Schut and Adams 2009) as well as the effect of environmental parameters on fermentation profiles and carbon metabolism (Desvaux et al. 2001, Cai et al. 2010, Rydzak et al. 2011, Willquist et al. 2011, Carere et al. 2014, Ciranna et al. 2014a). In pure culture systems the reduced microbial diversity eases the identification and the prediction of metabolic behavior in response to process parameters revealing important information regarding the conditions that promote high H_2 yield and production rate (Ljunggren et al. 2011a). In addition, metabolic engineering strategy can be used to improve the overall performance of pure strains (Hallenbeck and Ghosh 2012).

H_2-producing organisms can be enriched from different sources such as anaerobic sludge, municipal sewage sludge, compost and soil (Wang and Wan 2009). Compared to pure cultures, enriched microbial communities metabolize a wider spectrum of substrates including complex feedstocks, are more robust and adaptable towards fluctuations in the process and can be employed in non-aseptic conditions thus preventing expensive costs for feedstock sterilization (Kleerebezem and van Loosdrecht 2007, Brenner et al. 2008). The downside of employing undefined cultures for H_2 production is the need to suppress methanogenic activity by pretreating the inoculum (Zhu and Béland 2006, O-Thong et al. 2009) or operating under conditions inhibiting H_2-consuming microorganisms (Kraemer and Bagley 2007). In addition, the production yield by mixed cultures is generally low (<2.5 mol H_2/mol glucose) due to the large variety of metabolic activities resulting in the production of undesirable reduced end-products (Kleerebezem and van Loosdrecht 2007, Li and Fang 2007, Koskinen et al. 2008b). In contrast, these microbial communities generate H_2 at high rates (Hallenbeck 2009). Given their properties, mixed cultures are more suitable for H_2 production from inexpensive feedstocks (e.g. wastewaters and solid wastes), where H_2 is a value added product of the process and achieving high production yields is not essential (van Niel et al. 2011, Lin et al. 2012).

In order to overcome the shortcomings pertaining to both pure and undefined mixed cultures, co-cultures can be tailored by carefully selecting two or more microorganisms intentionally combined together. Stable *de novo* H_2-producing consortia can be established thanks to synergistic interactions based on complementary substrate utilization (Liu et al. 2008, Geng et al. 2010, Li and Liu 2012), O_2 scavenging (Yokoi et al. 1998), broadened optimal process conditions (Lu et al. 2007a) and excretion of stimulating and/or signaling compounds (Zeidan et al. 2010). In addition, several interspecies relationships could be engineered by genetic manipulation to control the

behavior of multiple interacting populations (Brenner et al. 2008), although similar strategies have never been used for H_2-producing consortia. Designed H_2-producing co-cultures should allow to enhance the performance of the H_2 production process by improving conversion efficiencies, increasing H_2 yield and production rate, and favoring bacterial growth and biomass formation. Although only few studies have been carried out to explore their potential for H_2 production, there is clear evidence that both mesophilic and thermophilic co-cultures can achieve better results than the mono-cultures with respect to different parameters (Elsharnouby et al. 2013, Kivistö et al. 2013a).

1.1.3. Feedstocks for fermentative H_2 production

Fermentative microorganisms can synthesize H_2 from a variety of substrates including carbohydrates, proteins and fats. However, practical H_2 fermentations are restricted to carbohydrate-rich materials, whereas protein- and fat-rich substrates yield limited amount of H_2 (Lay et al. 2003, Hallenbeck 2009). Hydrogen can be produced from a wide spectrum of carbohydrates from simple sugars to more complex plant-derived polysaccharide components. The majority of studies on H_2 production by dark fermentation have been conducted with model compounds, mainly mono- or disaccharides (e.g. glucose, xylose, maltose, sucrose and cellobiose), but also polysaccharides (e.g. starch, cellulose and xylan) and polyalcohols (e.g. glycerol) (Elsharnouby et al. 2013). While the use of simple sugars is not ideal for a cost-effective and sustainable process, it allows for more basic studies on the metabolism and physiology of H_2-producing microorganisms (Yoshida et al. 2006, Willquist and van Niel 2010, Zhou et al. 2013). Usually higher production yields can be achieved from simple carbohydrates allowing to test the limits of fermentative metabolism for a specific organism or community (Schröder et al. 1994, Zeidan and van Niel 2010). In addition, "synthetic wastes" are employed to probe and model the fermentation preferences of the bacteria among the different types of sugars in absence of inhibiting molecules usually present in real feedstocks (Table 5). Finally, the use of pure polymeric sugars as substrates can be useful to assess the ability of a microbial culture to convert complex feedstocks (e.g. lignocellulosic biomass) to H_2 (Table 5).

In order to be environmentally and economically sustainable dark fermentative H_2 production must rely on renewable substrates. The ideal feedstock needs to be inexpensive, derive from sustainable resources, require minimum pretreatment and contain sufficient concentration of fermentables so that conversion is energetically favorable (Hawkes et al. 2002). The potential feedstocks include biomass, agricultural waste, lignocellulosic products (wood and wood waste), wastewaters, solid wastes, biodiesel wastes (crude glycerol), aquatic plants, algae, agricultural and livestock effluents (Table 5). Strictly anaerobic bacteria are known to be able to simultaneously

hydrolyze lignocellulosic substrates and ferment the products of the breakdown to several end-products including H_2 (Lynd et al. 2002). Thus, plant-derived biomass can provide a suitable feedstock for fermentative H_2 production. Harvesting biohydrogen from treatment of organic wastes represents an attractive solution. Several wastewaters and solid wastes have been investigated as potential source of H_2 mainly employing undefined cultures (Table 5) (Li and Fang 2007). In recent years some other promising feedstocks including oil industry wastewaters, biodiesel wastes containing glycerol and microalgal biomass have received attention for their possible use for biohydrogen production (Table 5) (Elsharnouby et al. 2013). Depending on the raw material and the characteristics of microbial culture in the fermentation, the feedstock generally requires pretreatments including mechanical grounding (plant biomass), chemical and/or enzymatic hydrolysis (plant biomass), detoxification (plant biomass, crude glycerol) and nutritional balancing (industrial wastewaters) (Lin and Lay 2005, Mosier et al. 2005, Kapdan and Kargi 2006, Chong et al. 2009a). Applying pretreatment strategies and addition of expensive nutrients (e.g. yeast extract, buffers, salts and trace elements) impact the overall H_2 production costs (Kapdan and Kargi 2006). Therefore, to develop a cost-effective process based on dark fermentation the entire process should be carefully designed by selecting the most suitable combination of feedstock, pretreatment and organism(s).

Table 5. Examples of fermentative H_2 production from model substrates and real renewable feedstocks.

Organism/ Source of inoculum	Substrate	Operational condition	Reactor type	H_2 yield	Q_{H2}[a]	References
Model substrates						
Synthetic wastes						
Ci. sp. CMC-1	Gal/man/xyl	Batch[T]	SB	1.9[b]	ND	Mangayil et al. 2011
Ca. saccharolyticus	Glu/xyl	Batch[T]	CSTR	3.4[b]	13.0	de Vrije et al. 2009
T. neapolitana	Glu/xyl	Batch[T]	CSTR	3.3[b]	14.5	de Vrije et al. 2009
The. thermosaccharolyticum	Glu/xyl/ara	Batch[T]	SB	2.4[b]	12.7	Ren et al. 2010
Laboratory-scale CSTR	Glu/ara	Continuous[M]	EGSB	175[c]	5.0	Abreu et. al 2010
Waste activated sludge	Proteins	Batch[T]	UR	205[c]	ND	Xiao et al. 2014
Polymeric sugars						
Cl. sp. TCW1	Cellulose	Batch[T]	UR	2.0[b]	2.6	Lo et al. 2011
Cl. thermocellum	Cellulose	Continuous[T]	CSTR	1.7[b]	1.0	Magnusson et al. 2009
The. thermosaccharolyticum	Xylan	Batch[T]	SB	120[c]	0.5	Saripan and Reungsang 2013
Cl. thermocellum & *Cl. thermopalmarium*	Cellulose	Batch[T]	SB	1.4[b]	ND	Geng et al. 2010
Cl. thermocellum & *The. thermosaccharolyticum*	Cellulose	Batch[T]	SB	1.6[b]	ND	Liu et al. 2009
Enriched rotted wood crumbs	Cellulose	Continuous[M]	Flask	1.8[b]	1.3	Wang et al. 2011
Real feedstocks						
Agricultural wastes & lignocellulosic products						
Cl. butyricum	SBH	Batch[M]	SB	1.7[b]	ND	Pattra et al. 2008
Cl. butyricum	PRS	Batch[M]	SB	0.8[b]	1.2	Lo et al. 2010
Ca. bescii	USG	Batch[T]	CSTR	ND	ND	Basen et al. 2014
Ca. saccharolyticus	Energy plants	Batch[T]	SB	0.5-3.8[b]	ND	Ivanova et al. 2009
Ca. saccharolyticus	MH	Batch[T]	CSTR	3.4[b]	12.6	de Vrije et al. 2009
Ca. saccharolyticus	PSP	Batch[T]	CSTR	3.5[b]	16.4	Mars et al. 2010
Ca. saccharolyticus	CP	Batch[T]	CSTR	2.8[b]	19.0	de Vrije et al. 2010
Ca. saccharolyticus	WSH	Continuous[T]	CSTR	3.4[b]	9.6	Pawar et al. 2013
T. neapolitana	MH	Batch[T]	CSTR	3.2[b]	13.1	de Vrije et al. 2009
T. neapolitana	PSP	Batch[T]	CSTR	3.8[b]	12.5	Mars et al. 2010

Table 5. Continued

Organism/ Source of inoculum	Substrate	Operational condition	Reactor type	H_2 Yield	Q_{H2}[a]	References
Agricultural wastes & lignocellulosic products						
T. neapolitana	CP	Batch[T]	CSTR	2.7[b]	12.5	de Vrije et al. 2010
The. thermosaccharolyticum	CSH	Batch[T]	SB	2.4[b]	12.7	Ren et al. 2010
Mixed anaerobic sludge	SSS	Batch[M]	Flask	127[c]	ND	Shi et al. 2010
Enriched cracked cereal	PPL	Batch[M]	SB	45[c]	ND	Cui et al. 2010
Laboratory-scale CSTR	WSH	Continuous[T]	CSTR	178[c]	0.34	Kongjan et al. 2010
Wastewaters & solid wastes						
Cl. beijerinckii	FW	Batch[M]	SB	128[c]	4.8	Kim et al. 2008
Cl. bifermentans	WS	Batch[M]	SB	47[c]	ND	Wang et al. 2003
Cl. butyricum	POME	Batch[M]	SB	ND	46.2	Chong et al. 2009b
T. neapolitana	CWP	Batch[T]	SB	2.5[b]	0.94	Cappelletti et al. 2012
Fermented soybean-meals	BCW	Batch[M]	SB	2.5[b]	ND	Mizuno et al. 2000
Enriched heat-treated soil	APW	Batch[M]	SB	93[c]	ND	van Ginkel et al. 2005
Enriched heat-treated soil	MW	Batch[M]	SB	37[c]	ND	van Ginkel et al. 2005
Compost	BW	Batch[M]	SB	0.34[b]	5.7	Fan et al. 2006
POME treatment plant	POME	Batch[T]	ASBR	2.6[b]	1.1	O-Thong et al. 2008b
POME treatment plant	POME	Batch[T]	ASBR	ND	17.0	Prasertsan et al. 2009
Laboratory-scale CSTR	TW	Continuous[T]	CSTR	2.3[b]	21.0	Kim and Lee 2010
Municipal sewage	TW	Continuous[T]	MR	1.9[b]	34.0	Kim et al. 2011a
Municipal sewage	RWW	Continuous[T]	UAR	1.9[b]	3.8	Yu et al.2002
UASB-treating CS	CS	Continuous[T]	CSTR	57[c]	6.2	Luo et al. 2010
Municipal sewage	FW	Continuous[T]	CSTR	2.2[b]	1.8	Shin and Youn 2005
Biodiesel wastes						
Cl. pasteurianum	WG	Continuous[M]	CSTR	0.8[d]	7.4	Lo et al. 2013
En. aerogenes	WG	Batch[M]	SB	0.9[d]	ND	Jitrwung and Yargeau 2011
K. pneumoniae	WG	Batch[M]	SB	0.5[d]	17.8	Liu et al. 2007
Ha. saccharolyticus	WG	Batch[M]	SB	3.0[d]	ND	Kivistö et al. 2013b
T. neapolitana	WG	Batch[T]	SB	2.7[d]	ND	Ngo et al. 2011
Municipal sewage	WG	Batch[M]	SB	1.1[d]	ND	Mangayil et al. 2012
Microalgal biomass						
Cl. butyricum	SOB	Batch[M]	SB	113[c]	2.4	Batista et al. 2014
En. aerogenes	SOB	Batch[M]	SB	58[c]	1.0	Batista et al. 2014
T. neapolitana	CRB	Batch[T]	SB	2.5[b]	10.1	Nguyen et al. 2010
T. neapolitana	TWB	Batch[T]	CSTR	1.9[b]	0.80	Dipasquale et al. 2012
Municipal sewage	LJB	Batch[M]	SB, UR	28[c]	3.1	Park et al. 2009
Anaerobic digester	CSB	Batch[T]	Flask	2.7[b]	ND	Roy et al. 2014

ND, not determined; M, mesophilic condition; T, thermophilic condition

a) volumetric H_2 production rate (mmol H_2/l/h)

b) H_2 yield reported as mol H_2/mol hexose equivalent

c) H_2 yield reported as ml H_2/g substrate or COD

d) H_2 yield reported as mol H_2/mol glycerol

Substrate abbreviations: APW, apple-processing wastewater; ara, arabinose; BCW, bean curd waste; BW, brewery waste; CP, carrot pulp; CRB, *Chlamydomonas reinhardtii* biomass; CS, cassava stillage; CSB, *Chlorella sorokiniana* biomass; CSH, corn stalk hydrolysates; CWP, cheese whey powder; FW, food waste; glu, glucose; LJB, *Laminaria japonica* biomass; man, mannose; MH, miscanthus hydrolysates; MW, municipal wastewater; POME, palm oil mill effluent; PPL, pretreated poplar leaves; PRS, pretreated rice straw; PSP, potato steam peel; RWW, rice winery wastewater; SBH, sugarcane bagasse hydrolysates; SOB, *Scenedesmus obliquus* biomass; SSS, sweet sorghum stalk; TW, tofu wastewater; TWB, *Thalassiosira weissflogii* biomass; USG, untreated switchgrass; WG, waste glycerol; WS, wastewater sludge; WSH, wheat straw hydrolysates; xyl, xylose

Reactor type abbreviations: ASBR; anaerobic sequencing batch reactor; CSTR, continuous stirred-tank reactor; MR, membrane reactor; EGSB, expanded granular sludge bed; Flask, flask-type reactor; SB, serum bottles; UAR, up-flow anaerobic reactor; UR, unspecified reactor

1.2. HYDROGEN PRODUCTION BY THERMOPHILIC FERMENTATION

In the past decade thermophilic fermentative anaerobes have drawn the attention of the scientific community for their ability to efficiently generate H_2 from organic substrates. These organisms are classified based on their optimal growth temperature as (moderate) thermophiles (50 °C <T_{opt}< 64 °C), extreme thermophiles (65 °C <T_{opt}< 79 °C) and hyperthermophiles (T_{opt}> 80 °C) (Wagner and Wiegel 2008). As a matter of fact, biological H_2 production under thermophilic conditions possesses some advantages over operating the same process at mesophilic conditions. In order to establish an economically viable biological process for H_2 production the yield needs to be maximized. A recent survey on the status of biohydrogen production by dark fermentation shows that thermophilic strains outperform mesophilic strains when the H_2 yield is taken into account (Rittmann and Herwig 2012). In mesophilic anaerobic bacteria the yield of conversion of substrate to H_2 is normally limited by thermodynamic constraints and by branched metabolism leading to production of different by-products. In contrast, several studies on thermophilic pure cultures reported H_2 production yields from organic substrates up to the theoretical limit of 4 mol H_2/mol hexose (Table 6) (Schröder et al. 1994, Soboh et al. 2004, de Vrije et al. 2009, Zeidan et al. 2010). In addition, enriching mixed-population at high temperature decreases the microbial and metabolic diversity allowing for the selection of more efficient H_2 producers (Shin et al. 2004, Karadag and Puhakka 2010, Orlygsson et al. 2010).

Several thermophiles exhibit an extensive array of glycoside hydrolases resulting in a superior hydrolytic capacity towards recalcitrant lignocellulosic biomass. Generally, the hydrolysis can occur via two different strategies: in *Cl. thermocellum*, for example, a cell surface-bound multienzymatic complex called cellulosome degrades the cellulose, whereas in organisms like *Caldicellulosiruptor* spp. and *Thermotoga* spp. (hemi)cellulases are secreted extracellularly to hydrolyze the substrate (Blumer-Schuette et al. 2008, VanFossen et al. 2008, Blumer-Schuette et al. 2013). The ability of these organisms to simultaneously degrade lignocellulosic substrates and ferment the products of the breakdown to H_2 in the same "pot" makes them good candidates for fermentative H_2 production by consolidated bioprocess (Lynd et al. 2002). Furthermore, the thermophilic fermentative H_2 production provides process-related benefits such as increased substrate solubility, lower viscosity, better mixing, reduced risk of contamination by H_2-consuming microorganisms and no need for cooling heat-treated feedstocks (Wiegel et al. 1985, van Niel et al. 2011).

Table 6. Effect of fermentation temperature on H_2 yield of pure cultures grown with model substrate.

Organism	T_{opt}	Substrate	Operational conditions	H_2 yield[a]	References
Mesophiles					
Cl. acetobutylicum	34	Glucose	Fed batch	2.0	Chin et al. 2003
Cl. beijerinckii	35	Glucose	Batch	2.5	Pan et al. 2008
Ci. sp. Y19	36	Glucose	Batch	2.5	Oh et al. 2003
E. coli SR15	37	Glucose	Fed batch	1.8	Yoshida et al. 2006
Ha. saccharolyticus	37	Glucose	Batch	2.4	Kivisto et al. 2010
Thermophiles					
Cl. thermocellum	60	Cellobiose	Continuous	3.5	Bothun et al. 2004
T. elfi	65	Glucose	Batch	3.3	van Niel et al. 2002
C. celer	67	Glucose	Batch	3.5	Ciranna et al. 2012
Cal. subterraneus	75	Glucose	Batch	4.0	Soboh et al. 2004
T. neapolitana	77	Glucose	Batch	3.9	d'Ippolito et al. 2010
Ca. saccharolyticus	70	Glucose	Continuous	4.0	de Vrije et al. 2007
Ca. owensensis	70	Glucose	Batch	4.0	Zeidan and van Niel 2010
Ca. kristjanssonii	70	Glucose	Batch	3.5	Zeidan and van Niel 2009
T. maritima	80	Glucose	Batch	4.0	Schroder et al. 1994
Thc. onnurineus	80	Starch	Batch	3.1	Bae et al. 2012
Thc. kodakaraensis	88	Starch	Continuous	3.3	Kanai et al. 2005
P. furiosus	100	Glucose	Batch	3.5	Kengen et al. 1994

a) mol H_2/mol hexose equivalent

1.2.1. Fermentative metabolism of thermophilic H_2 producers

In the last decades the increasing general interest towards thermophilic anaerobes for their potential biotechnological application resulted in a thorough investigation of the physiology and metabolism of these species, initially mainly based on biochemical and fermentation studies and nowadays embracing the power of "omics" and metabolic engineering. Among the thermophilic H_2 producers, some species (i.e. *Ca. saccharolyticus*, *T. maritima*, *Cl. thermocellum*, *P. furiosus* and *Cal. subterraneus* subsp. *tengcongensis*) have emerged as model organisms for their peculiar properties. Thanks to the studies on these organisms, some light has been shed on their fermentative and energy metabolism, and how they are linked to their ability of producing H_2 at high yields.

Carbohydrate metabolism

Carbohydrate uptake in thermophilic anaerobes can be mediated by ATP-binding Cassette (ABC)-type transporters and phosphotransferase system (PTS) transporters. Both systems are energy-demanding since both rely on the consumption of high-energy compounds (2 ATP or one phosphoenolpyruvate per carbohydrate molecule, respectively) to transport and phosphorylate sugars. Most of these organisms utilize ABC-type sugar uptake systems as the entry-point for the carbohydrates into the cell (Nochur et al. 1992, Nelson et al. 1999, Robb et al. 2001, Wang et al. 2004, van Fossen et al. 2009). These transporters have high affinity for the substrate (Koning et al. 2001) and a broader specificity towards multiple sugars, albeit with varying affinities (van Fossen et al. 2009). On the other hand, they require a higher energy investment (2 ATP per sugar). From an ecological point of view, the abundance of these transport systems

in thermophilic organisms suggests that they play a major role in sugar utilization in the nutrient-poor extreme environments in which these organisms thrive (Koning et al. 2001). Although less in number, PTS-type transporters have been identified in few thermophilic H_2 producers (Wang et al. 2004, van Fossen et al. 2009).

Phosphorylated sugars are converted to pyruvate via Embden-Meyerhof-Parnas (EMP) pathway, Entner-Doudoroff (ED) pathway or pentose phosphate pathway (PPP). In thermophilic anaerobes the common route for hexose catabolism is the EMP pathway (van Niel et al. 2011), although some hyperthermophilic archaea and bacteria can employ both EMP and ED pathways (Selig et al. 1997). The classical EMP pathway yields 2 ATP and 2 NADH per mole of glucose metabolized. Indeed, 2 ATP are consumed in the preparatory phase (glucose to glyceraldehyde-3-phosphate) and 4 ATP are produced during the payoff phase (glyceraldehyde-3-phosphate to pyruvate) by phosphoglycerate kinase and pyruvate kinase, while 2 NADH are generated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Figure 3). Some thermophilic anaerobes (both bacteria and archaea) utilize a modified EMP pathway. In fact, some variations can be observed regarding the energy currencies employed or produced by phosphorylating enzymes (i.e. hexose kinases and phosphoenolpyruvate/pyruvate kinases) (Figure 3). For example, in *Thermococcales* (e.g. *P. furiosus* and *Thc. kodakarensis*) sugar kinases are ADP-dependent instead of being ATP-dependent and phosphoenolpyruvate (PEP) is converted to pyruvate by either a PEP synthase or a pyruvate kinase (Figure 3) (Verhees et al. 2003, Sakuraba et al. 2004, Siebers and Schönheit 2005). In *T. maritima* and *Ca. saccharolyticus* pyrophosphate (PPi) plays a significant role as an energy carrier in the glycolytic reactions along with ATP. These organisms utilize a PPi-dependent phosphofructokinase (PPi-PFK) in addition to the ATP-dependent phosphofructokinase (ATP-PFK) (Ding et al. 2001, Bielen et al. 2010). Moreover, *Ca. saccharolyticus* can also catalyze the conversion of PEP to pyruvate by a pyruvate phosphate dikinase (PPDK) yielding ATP from AMP and PPi (Bielen et al. 2010). Another important variation of the classical EMP pathway can be observed in archaea, where the oxidation of glyceraldehyde-3-phosphate (GAP) is mediated by the ferredoxin-dependent GAP oxidoreductase (GAPOR) instead of the conventional NAD-dependent GAPDH (Figure 3) (Verhees et al. 2003, Sakuraba et al. 2004, Siebers and Schönheit 2005). This reaction converts GAP directly to 3-phosphoglycerate skipping the ATP production step by substrate level phosphorylation. As a result of these modifications, the glycolysis in *Thermococcales* yields from 0 to 2 ATP (depending on the need to regenerate ADP for hexose phosphorylation by the reaction ATP + AMP → 2ATP) and 4 molecules of reduced ferredoxin (Fd_{red}), while in *Ca. saccharolyticus* and *T. maritima* from 2 to 3 ATP are produced (depending on the use of PPi in the reaction catalyzed by PFK) along with 2 NADH. In fermentative thermophilic bacteria pentose sugars are metabolized via the non-oxidative PPP entering in the glycolysis as fructose 6-phosphate (F6P) and GAP (van Niel et al. 2011).

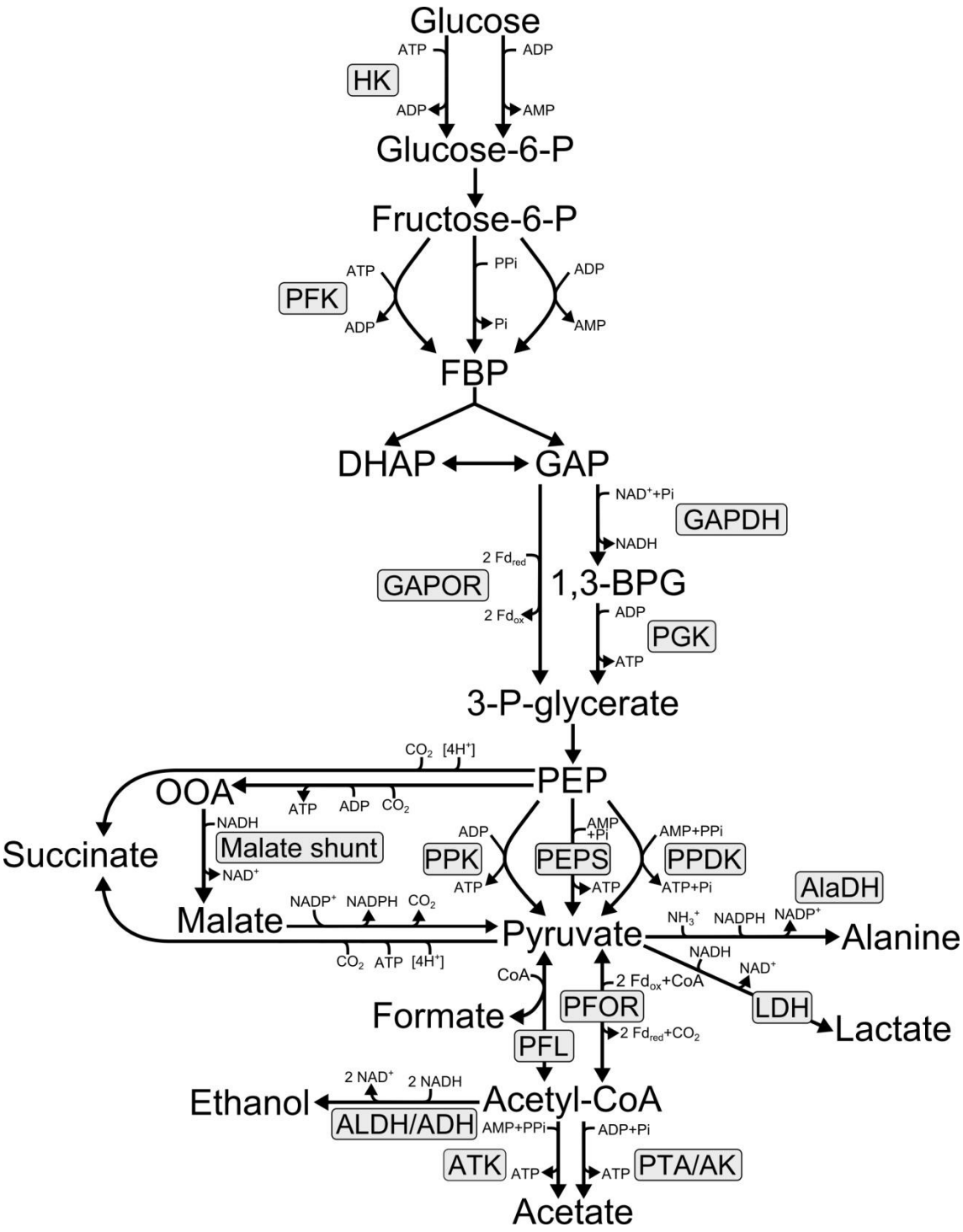

Figure 3. Metabolic pathways of glycolysis, pyruvate catabolism and end-product synthesis in model thermophilic H_2 producers. Abbreviations: 1,3-BPG, 1,3-bisphosphoglycerate; Acetyl-CoA, acetyl coenzyme A; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CoA, Coenzyme A; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-biphosphate, Fd_{ox}, ferredoxin (oxidized form); Fd_{red}, ferredoxin (reduced form); GAP, glyceraldehyde-3-phosphate; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form), OOA, oxaloacetate; PEP, phosphoenolpyruvate; Pi, orthophosphate; PPi, pyrophosphate. The abbreviations of the enzymes are reported in Table 7.

Pyruvate, the final product of the glycolysis, is a key intermediate of the central carbon metabolism and its fate strongly affects the end-product profile. For a biotechnological exploitation of microbial fermentative metabolism it is crucial to understand the factors that regulate carbon and electron fluxes at this metabolic branch point and therefore the final distribution of metabolites to channel the metabolic flux towards the desired product. Thermophilic H_2 producers display a limited number of reactions for pyruvate utilization compared to mesophiles resulting in a reduced variety of end-products (Verhaart et al. 2010, Bielen et al. 2013a). For maximal H_2 synthesis, pyruvate should be converted to acetate via PFOR (Figure 3) (Verhaart et al. 2010, van Niel et al. 2011). In addition to those produced during the glycolysis (in form of NADH or Fd_{red}), this route provides extra reducing equivalents (in form of Fd_{red}) to feed the hydrogenogenic reactions, whereas in the alternative reaction catalyzed by PFL the reducing equivalents remain with the product formate. The conversion of pyruvate to acetate is also energetically convenient since an extra ATP is generated when acetate is produced from acetyl-CoA (Figure 3). A number of pathways departing from the pyruvate node are used to reoxidize NADH and control the redox state in response to environmental changes (Table 2, Table 7, Figure 3). In thermophilic H_2 producers the most common products of these reactions are lactate, ethanol and alanine, although synthesis of other compounds (e.g. butyrate, succinate, etc.) in lower amounts is possible.

H_2 synthesis, hydrogenases and electron shuttling

Hydrogenases are complex oxygen-sensitive metalloenzymes typically constituted by multiple subunits that catalyze the reversible reduction of proton to molecular H_2 (Eq. 2). The direction of the reaction depends on the redox potential of the components interacting with the enzyme (Vignais and Billoud 2007). In microorganisms unable to utilize external electron acceptors H_2ases are used to dispose the reducing power accumulated during catabolism by reduction of protons to molecular H_2. In contrast, the oxidation of molecular H_2 provides several organisms (e.g. methanogens, sulfate reducers and photosynthetic bacteria) with useable reductants needed in energy-generating steps. Due to their great scientific and technological interest, H_2ases have been extensively studied and reviewed (Vignais et al. 2001, Böck et al. 2006, Fontecilla-Camps et al. 2007, Meyer 2007, Vignais and Billoud 2007, Eberly and Ely 2008, Jenney Jr. and Adams 2008, Heinekey 2009, Tard and Pickett 2009, Calusinska et al. 2010, Barton et al. 2010, Kim and Kim 2011, Mulder et al. 2011). They are classified based on their catalytic metal center in three phylogenetically distinct groups: NiFe-H_2ases, FeFe-H_2ases and FeS cluster-free H_2ases (Vignais and Billoud 2007). NiFe-H_2ases are widely distributed among archaea and bacteria, whereas FeFe-H_2ases are restricted to bacteria and lower eukaryotes. The latter group, instead, is found only in methanogenic archaea and therefore it will not be further discussed in this thesis.

Table 7. Metabolic features of C. celer *and other model thermophilic H_2 producers (modified and continued from Chou et al. 2008 and Pawar and van Niel 2013).*

Organism	Substrates metabolized	CCR	Auxotrophy to AA	Key glycolytic enzymes	Enzymes in pyruvate catabolism	Electron carriers	H_2ases & electron shuttling systems	Alternative reductant sink to H_2	References
C. celer	C_6 sugars, maltose, sucrose, starch	ND	Yes	ATP-HK, ATP-PFK, GAPDH, PGK, PPK, PPDK, MS	PFL, PFOR, ALDH/ADH, PTA/AK	Fd, NADH	$HYD^{A1\ TR(M3)}$, MBH^{G4}, $HYA^{G1,}$ $Sensory^{D\ M2e}$, MBX, NFN	Formate, ethanol, butyrate	Ciranna et al. 2013, Calusinska et al. 2010
Ca. saccharolyticus	C_6 and C_5 sugars, cellulose, xylan, pectin, α-glucan, β-glucan, guar gum	No	No	ATP-HK, ATP-PFK, PPi-PFK, GAPDH, PGK, PPK, PPDK, pMS	PFOR, LDH, ADH, PTA/AK	Fd, NADH	$HYD^{A1\ TR(M3)}$, $Sensory^{D\ M2e}$, ECH^{G4}, NFN	Lactate, ethanol, succinate	van de Werken et al. 2008, Bielen et al. 2010, Calusinska et al. 2010, Carere et al. 2013
T. maritima	C_6 and C_5 sugars, cellobiose, starch, cellulose, xylan	Yes	No	ATP-HK, ATP-PFK, PPi-PFK, GAPDH, PGK, PPK, PPDK	PFOR, LDH, AlaDH, ADH, PTA/AK	Fd, NADH	$HYD^{TTH,}$ $Sensory^{CD(M2f)}$, MBX, RNF NFN	Lactate, alanine	Ding et al. 2001, Schäfer and Schönheit 1991, Schut and Adams 2009, Calusinska et al. 2010, Carere et al. 2013
Cal. subterraneus subsp. *tengcongensis*	C_6 sugars, cellobiose, maltose, lactose, starch	Yes	Yes	ATP-HK, ATP-PFK, GAPDH, PGK, PPK, PPDK	PFOR, ADH, PTA/AK	Fd, NADH	$HYD^{A1\ TR(M3)}$, $Sensory^{D\ M2e}$, ECH^{G4}, NFN	Ethanol	Soboh et al. 2004, Calusinska et al. 2010, Carere et al. 2013
Cl. thermocellum	C_6 sugars, starch, cellulose	Yes	No	ATP-HK, ATP-PFK, GAPDH, PGK, PPDK, MS	PFL, PFOR, LDH, ALDH/ADH, PTA/AK, ATK	Fd, NADH	$Uptake^{A\ D(M2g)}$ $HYD^{A1/A8\ TR(M3)}$, $Sensory^{CD(M2f)}$, $Sensory^{D\ M2e}$ ECH^{G4}, RFN, NFN	Formate, ethanol, lactate	Calusinska et al. 2010, Carere et al. 2013, Rydzak et al. 2014
P. furiosus	C_6 sugars, maltose, cellobiose, β-glucans, starch	No	Yes	ADP-HK, ADP-PFK, GAPOR, PPK, PEPS	PFOR, AlaDH, ADH, ATK	Fd	$Uptake^{G3}$ MBH^{G4}, MBX, NFN	Alanine, ethanol	Robb et al. 2001, Verhees et al. 2003, Sapra et al. 2003, Sakuraba et al. 2004, Siebers and Schönheit 2005, Schut et al. 2007, Carere et al. 2013

Superscripts indicate phylogenetic cluster groupings of the H_2ases according to Calusinska et al. 2010

Abbreviations: AA, amino acids; ADP-HK, ADP-dependent hexokinase; ADP-PFK, ADP-dependent phosphofructokinase; AlaDH, alanine dehydrogenase; ALDH/ADH, aldehyde/alcohol dehydrogenase; ATK, acetate thiokinase; ATP-HK, ATP-dependent hexokinase; ATP-PFK, ATP-dependent phosphofructokinase; CCR, carbon catabolite repression; Fd, ferredoxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAPOR, glyceraldehyde-3-phosphate oxidoreductase; HYD, (bifurcated) NADH-dependent FeFe-H_2ase; LDH, lactate dehydrogenase; MBH & ECH, energy-conserving membrane-bound NiFe-H_2ase; MBX, energy-conserving ferredoxin:$NAD(P)^+$ oxidoreductase; NADH, nicotinamide adenine dinucleotide; ND, not determined; NFN, NADH-dependent ferredoxin:$NADP^+$ oxidoreductase; (p)MS, (partial) malate shunt; PEPS, phosphoenolpyruvate synthase; PFL, pyruvate formate lyase; PGK, phosphoglycerate kinase; PPDK, pyruvate phosphate dikinase; PPi-PFK, PPi-dependent phosphofructokinase; PPK, pyruvate phosphate kinase; PFOR, pyruvate:ferredoxin oxidoreductase; PTA/AK, phosphotransacetylase/acetate kinase; RNF, proton-translocating ferredoxin:NAD^+ oxidoreductase; Sensory, sensory FeFe-H_2ase; Uptake, NADPH-dependent NiFe- or FeFe-H_2ase

FeFe-H_2ases are highly modular enzymes and their classification relies mainly on the distribution of the domains within the subunits (Meyer 2007, Calusinska et al. 2010). They exist as monomeric enzymes or multimeric complexes (dimers, trimers or tetramers) usually localized in the cytoplasm. In general, FeFe-H_2ases contain a catalytic core (H cluster) in the catalytic subunit, along with numerous domains accommodating iron-sulfur clusters (2[4Fe4S], [4Fe4S], [2Fe2S], etc.) located in the catalytic subunit and/or accessory subunits. In thermophilic H_2 producers several FeFe-H_2ases have been identified on the basis of biochemical characterization and/or sequence alignment analysis. The majority of thermophilic anaerobic bacteria possess heterotetrameric and/or heterotrimeric FeFe-H_2ases belonging to A1 group exhibiting a TR(M3) modular structure (Table 7) (Calusinska et al. 2010, Carere et al. 2012). Given the presence of NADH-binding domains in the accessory subunits, these H_2ases are thought to be NADH-dependent. Two of these enzymes have been characterized at biochemical level. The heterotetrameric FeFe-H_2ase of *Cal. subterraneus* subsp. *tengcongensis* utilizes NADH as an electron donor for proton reduction (Soboh et al. 2004), whereas the heterotrimeric FeFe-H_2ase (also called bifurcating H_2ase) of *T. maritima* simultaneously oxidizes Fd_{red} and NADH in a 1:1 ratio to H_2 (Schut and Adams 2009). Due to the high level of homology shared by the α, β and γ subunits of the two enzymes and the thermodynamic limitation of deriving H_2 directly from NADH (see section 3.2), it was suggested that also heterotetrameric FeFe-H_2ases should possess a bifurcating activity (Schut and Adams 2009). Given the thermodynamic advantage of driving H_2 synthesis through bifurcating H_2ases (see section 3.2), it is tempting to predict the same activity for all the H_2ases belonging to this group simply on the basis of sequence homology. However, more heteromultimeric FeFe-H_2ases should be characterized in order to validate this hypothesis.

Other FeFe-H_2ases identified in thermophilic H_2 producers include heterodimeric NADPH-dependent FeFe-H_2ases, heterodimeric Fd-dependent FeFe-H_2ases containing PAS/PAC sensory domains and monomeric sensory H_2ases (Table 7). In *Cl. thermocellum* a heterodimeric NADPH-dependent FeFe-H_2ases (Group A, D(M2g)) is suggested to derive electrons from H_2 to reduce $NADP^+$ to NADPH possibly supplying the cell with reducing equivalents for biomass synthesis and NADPH-dependent ethanol production (Rydzak et al. 2014). A dimeric Fd-dependent FeFe-H_2ases (group C, D(M2f)) was found to be the major responsible for H_2 production in *The. saccharolyticum* (Shaw et al. 2009). Interestingly, one subunit of this H_2ase contains a PAS/PAC sensory domain known to be part of two-component kinase signaling systems and thus possibly takes part in the regulation of cellular redox state (Taylor and Zhulin 1999). Genes encoding for monomeric sensory H_2ases (group D, M2e) have been identified upstream the A1 group multimeric FeFe-H_2ases along with histidine kinase

protein suggesting a possible regulatory role of these H_2ases (Calusinska et al. 2010, Carere et al. 2012, Verbeke et al. 2013).

NiFe-H_2ases possess at least two subunits: a large catalytic subunit containing the NiFe active center and a small subunit containing FeS clusters mediating the transfer of electrons from/to the electron donor/acceptor. Additionally, several accessory subunits can participate in the formation of larger complexes. NiFe-H_2ases are classified in four main groups (Vignais and Billoud 2007). Thus far, in thermophilic H_2 producers only NiFe-H_2ases belonging to group 3 and group 4 have been characterized. Cytosolic bidirectional heteromultimeric NiFe-H_2ases belonging to group 3 are found in thermophilic archaea such as *Thermococcales* (e.g. *Pyrococcus* spp. and *Thermococcus* spp.). In *P. furiosus* and *Thc. kodakarensis* these H_2ases are NADP(H)-dependent and are involved in H_2 recycling to provide NADPH for biosynthesis, thus functioning as uptake H_2ases (van Haaster et al. 2008, Kanai et al. 2011). H_2-evolving, energy-conserving, membrane-associated multimeric NiFe-H_2ases (group 4) are common in thermophilic H_2-producing organisms (Vignais and Billoud 2007). These H_2ases consist of six or more subunits encoded by genes clustered into a single operon. The six core subunits have an high level of homology with six subunits (NuoBCDHIL) of the energy conserving NADH:quinone oxidoreductase (complex I) in *E. coli* (Hedderich 2004). The energy-conserving H_2ase (ECH) from *Cal. subterraneus* subsp. *tengcongensis* (Soboh et al. 2004) and the membrane-bound H_2ase (MBH) from *P. furiosus* (Sapra et al. 2003) couple the Fd_{red}-dependent reduction of protons with the electrogenic translocation of ion across the membrane. The resulting "motive force" can be used to generate ATP via a membrane-bound ATP synthase. Therefore, this mechanism for energy conservation can be considered as a proton respiration event.

Electron transfer can occur, not only between Fd and/or NAD(P)H and proton/H_2, but also directly between Fd and NAD(P)H. Several thermophilic H_2 producers can catalyze this reaction via three types of ferredoxin:$NAD(P)^+$ oxidoreductases (FNOR). The Rnf-like FNOR is a membrane-bound enzymatic complex that can couple the electron transfer from Fd_{red} to NAD^+ with electrogenic translocation of ion across the membrane. The energy stored in the electrochemical ion gradient can then drive ATP synthesis via membrane-bound ATP synthase (Müller et al. 2008). It has been suggested that in *T. maritima* this enzymatic complex could have a role in maintaining an appropriate Fd_{red}/NADH ratio for the bifurcating FeFe-H_2ase and other cell processes (Schut and Adams 2009). MBX is a membrane-bound ferredoxin:$NADP^+$ oxidoreductase complex showing high sequence identity and similar gene organization to MBH. However, this complex does not possess H_2ase activity, but rather transfers electron from Fd_{red} to $NADP^+$ and conserves energy by pumping protons (Schut et al. 2007). The function of MBX in *P. furiosus* has been studied in relation to sulfur metabolism and its role in hydrogenogenesis is not clear. The NADH-dependent ferredoxin:$NADP^+$

oxidoreductase (NFN) is a heterodimeric cytosolic electron-bifurcating enzyme that catalyzes the reduction of $NADP^+$ to NADPH by using both Fd_{red} and NADH as electron donors (Fd_{red} + $2NADP^+$ + NADH + H^+ ↔ Fd_{ox} + NAD^+ + 2NADPH). Although several thermophilic H_2 producers harbor this enzyme, its role in these organisms has not been investigated in detail. Perhaps, it could be the yet unidentified source of NADPH for biomass synthesis in some fermentative organisms such as *Ca. saccharolyticus*.

Overall, each thermophilic H_2-producing organism is equipped with multiple and different H_2ases and/or oxidoreductases (Table 7) characterized by different electron carrier specificities, suggesting that the electron flow to H_2 or other reduced end-products might be not as straightforward as once thought.

1.2.2. Thermodynamics of H_2 synthesis

The complete oxidation of glucose to H_2 and CO_2 (Eq. 3) is not thermodynamically favorable under standard conditions ($\Delta G^{0\prime}$= +3.2 kJ/mol) and thus requires an extra energy input to proceed. On the other hand, the conversion of glucose to a variety of reduced metabolic products (e.g. organic acids and alcohols) is a more thermodynamically favorable reaction ($\Delta G^{0\prime}$< 0 kJ/mol) and can support microbial growth (Thauer et al. 1977). Further reduction of these molecules to H_2 by fermentative organisms in monocultures is highly unfavorable under standard conditions ($\Delta G^{0\prime}$> 0 kJ/mol). As a consequence, in dark fermentation glucose oxidation is thermodynamically limited, yielding a theoretical maximum of 4 H_2 moles per mole of glucose when acetate and CO_2 are the other end-products of the fermentation (Eq. 4) (Levin et al. 2004, Hallenbeck 2009). This yield can be achieved only when all the reducing equivalents generated during sugar catabolism (i.e. NADH and Fd_{red}) are disposed via proton reduction to molecular H_2.

Table 8. Gibbs free energy values for different fermentative reactions (Verhaart et al. 2010).

Fermentative reaction	**$\Delta G^{0\prime}$ kJ/mol**
$NADH + H^+ + pyruvate^- \rightarrow NAD^+ + lactate^-$	−25.0
$2NADH + 2H^+ + acetyl\text{-}CoA \rightarrow 2NAD^+ + ethanol + CoA$	−27.5
$NADH + H^+ + pyruvate^- + NH_4^+ \rightarrow NAD^+ + alanine + H_2O$	−36.7
$NADH + H^+ \rightarrow NAD^+ + H_2$	+18.1
$2Fd_{red} + 2H^+ \rightarrow 2Fd_{ox} + H_2$	+3.1

However, reoxidation of reduced electron carriers can be achieved by different reactions whose likelihood to occur depends on the standard Gibbs free energy change ($\Delta G^{0\prime}$) of the individual conversions (Table 8). H_2-evolving reactions are endergonic under standard conditions, thus they are not thermodynamically favorable compared to other reactions. Since $\Delta G'$ is a function of dissolved H_2 concentration ($[H_2]_{aq}$) (Eq. 5), this must be at low level for the H_2-evolving reactions to spontaneously occur.

$$\Delta G' = \Delta G°' + RT * \ln \frac{[NAD^+ \text{ or } Fd_{ox}]^a * [H_2]^b}{[NADH \text{ or } Fd_{red}]^c * [H^+]^d} \quad (5)$$

(a-d: stoichiometric reaction coefficients)

In natural environments this condition is guaranteed by the activity of H_2-consuming microorganisms such as methanogens or sulfate reducers (Stams 1994, van Niel et al. 2011). However, under experimental conditions supersaturation of the aqueous phase with H_2 can easily occur due to limitation of liquid-to-gas mass transfer rate creating challenging conditions for maximal H_2 production (Kraemer and Bagley 2006, Ljunggren et al. 2011a).

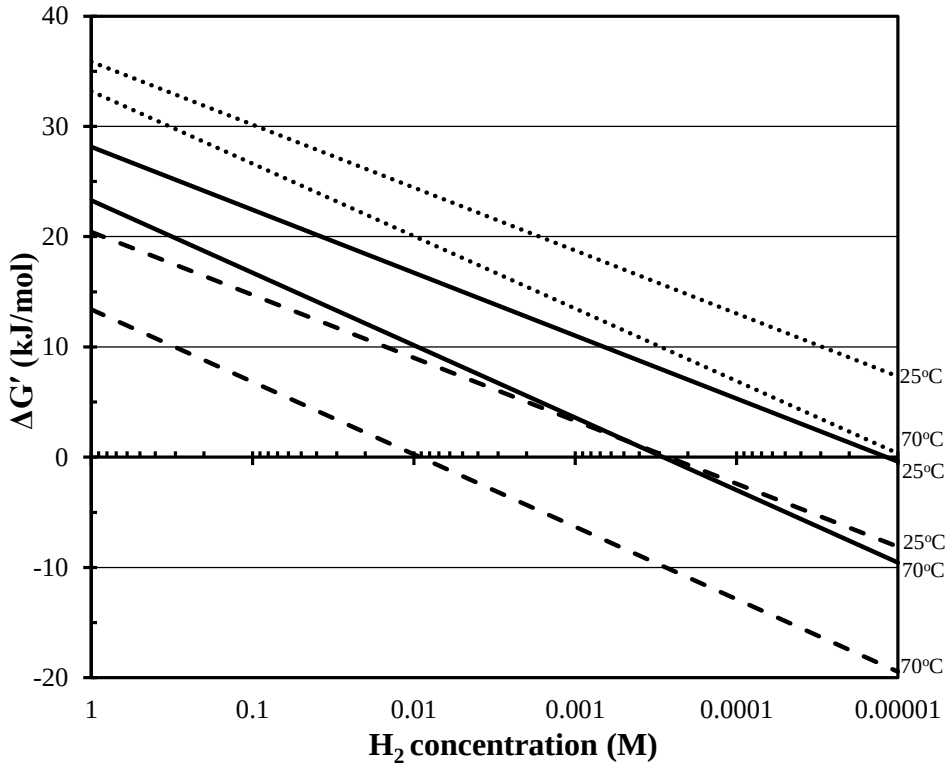

Figure 4. ΔG' of H_2 formation from NADH (dotted line), reduced ferredoxin (dashed line) and by bifurcating H_2ase (50 % NADH and 50 % Fd_{red}) (solid line) at 25 °C and 70 °C (modified from Bielen et al. 2013).

In general, protons are poor electron acceptors ($E^{0'}$= -414 mV) and can be reduced only by strong reducing agents (Thauer et al. 1977). The midpoint redox potentials of Fd_{red}/Fd_{ox} and $NADH/NAD^+$ are -400 mV and -320 mV, respectively (Thauer et al. 1977). Since $NADH/NAD^+$ has a higher formal potential than H^+/H_2, under standard conditions H_2 synthesis from NADH is less energetically favorable compared to the one from Fd_{red} (Table 8). Consequently, according to Eq. 5 a lower $[H_2]_{aq}$ is required for NADH-dependent H_2 formation to become exergonic (at 25 °C, 0.5 µM for NADH vs. 0.3 mM for Fd_{red}) (Figure 4) (Bielen et al. 2013a). In addition, the $\Delta G'$ of these reactions decreases when the temperature increases enhancing the effect of reduced $[H_2]_{aq}$. Thus,

at thermophilic conditions H_2 formation becomes exergonic at higher $[H_2]_{aq}$ (at 70 °C, 8.8 μM for NADH and 9.1 mM for Fd_{red}) (Figure 4). Due to the thermodynamic barrier associated to NADH-dependent H_2 synthesis, in fermentative mesophiles NADH reoxidation occurs by alternative reactions (e.g. synthesis of ethanol, lactate, butanol and acetone) whose thermodynamics is more favorable and independent of $[H_2]_{aq}$ (Table 8). As a result in these organisms the maximum H_2 yield is 2 mol H_2/mol hexose. In contrast, H_2 yield close to the theoretical limit has been observed in several thermophilic organisms, indicating that complete disposal of reducing equivalents via proton reduction is possible. Yet, experimental data shows that during anaerobic fermentations $[H_2]_{aq}$ can range from 0.4 to 2.2 mM (Kraemer and Bagley 2006, Ljunggren et al. 2011a), well above the critical $[H_2]_{aq}$ for exergonic NADH-dependent H_2 formation.

A plausible explanation for the ability of some fermentative thermophiles to overcome the thermodynamic limitation associated to direct H_2 production from NADH might be found in recently characterized energy-conserving enzymatic systems (Buckel and Thauer 2013). In particular, a novel type of FeFe-H_2ase characterized in some anaerobic bacteria, including *T. maritima* (Schut and Adams 2009), *Moorella thermoacetica* (Huang et al. 2012) and *Acetobacterium woodii* (Schuchmann and Müller 2012), couples the exergonic oxidation of Fd_{red} to drive the unfavorable oxidation of NADH to produce H_2. The reaction catalyzed by this bifurcating FeFe-H_2ase ($NADH + 2Fd_{red} + 3H^+ \rightarrow 2H_2 + NAD^+ + 2Fd_{ox}$) is more energetically favorable compared to the direct NADH oxidation (Figure 4). Many thermophiles and, surprisingly, also several mesophiles are predicted to encode this H_2ase on the basis of sequence homology (Table 7) (Schut and Adams 2009). However, it remains to be seen whether this energy conservation method is as widespread among fermentative anaerobes as the bioinformatics analysis suggests. Alternatively, electrons from NADH can be used for H_2 production if an energy input is provided to drive the reaction (Verhaart et al. 2010, Biegel et al. 2011, Bielen et al. 2013a). This could be achieved by reverse electron transport based on membrane-bound FNORs that exploits a proton or ion gradient to transfer electrons from NADH to Fd_{red}, which in turn is used for Fd-dependent H_2 production. Such strategy, however, has never been characterized in thermophilic H_2 producers and some of them (e.g. *Ca. saccharolyticus* and *Cal. subterraneus* subsp. *tengcongensis*) do not even harbor genes encoding membrane-bound NFOR complexes (Table 7).

1.2.3. H_2 inhibition and metabolic shift

The thermodynamics of the fermentative reactions is crucial to determine how reductants are disposed in the cell and, as discussed above, concentration of H_2 in the system is an important part of the equation. The inhibitory effect on microbial growth

and H_2 production caused by H_2 build-up during anaerobic fermentation is well documented (Schäfer and Schönheit 1991, Schröder et al. 1994, Van Niel et al. 2003, Soboh et al. 2004, Johnson et al. 2006). Investigations of this phenomenon most often rely on the quantification of the H_2 concentration in the gas phase (P_{H2}) to measure H_2 tolerance due to its more practical determination. A series of studies has been conducted to find a relation between P_{H2} and inhibition of H_2 production in *Ca. saccharolyticus* (Van Niel et al. 2003, Willquist and van Niel 2010, Willquist et al. 2010, Ljunggren et al. 2011a, van Niel et al. 2011, Willquist et al. 2011). The results showed that the idea of determining a general P_{H2} threshold for H_2 inhibition does not seem to be feasible. In fact, multiple variables (e.g. the organism and its metabolic potential, redox system, growth phase, carbon and energy source, intracellular redox and energetic states, ability to use alternative electron sink, osmolality, reactor design, stirring rate and other environmental parameters) seem to affect tolerance towards H_2 accumulation even within the same species. These factors can also be interdependent (e.g. growth phase and intracellular redox and energetic states) making the global understanding of H_2 inhibition more complex (Bielen et al. 2010, Willquist and van Niel 2010). In addition, using P_{H2} as a parameter to quantify H_2 tolerance may lead to inaccurate results because H_2 can easily supersaturate the liquid phase and its concentration can be far from the equilibrium (up to 35 times) (Kraemer and Bagley 2006, Ljunggren et al. 2011a). Thus, relying on P_{H2} measurements can lead to underestimation of the H_2 in the system, whereas $[H_2]_{aq}$ would be a more appropriate and accurate parameter and would facilitate comparison among different studies (Willquist et al. 2010, Bielen et al. 2013a).

Regardless of the method used to quantify H_2 in the system, it is demonstrated that excessive H_2 build-up triggers a metabolic shift towards production of other reduced end-products (e.g. ethanol, lactate, alanine, etc.) and in some cases completely inhibits growth. Both consequences are the result of an altered redox state. The dissolved H_2 concentration is a function of H_2 productivity and liquid-to-gas mass transfer rate, the former being dependent on growth rate and/or substrate concentration and the latter on gas stripping and stirring rates (Ljunggren et al. 2011a). At elevated $[H_2]_{aq}$ H_2 formation by H_2ases is inhibited and NADH can not be reoxidized through proton reduction. This leads to an increase of the intracellular NADH/NAD^+ ratio which in turn affects both growth rate and fermentation profile. The activity of GAPDH, the key enzyme in the glycolytic pathway, is known to be negatively affected by high NADH/NAD^+ resulting in a decrease of glycolytic flux and consequently of growth rate and substrate consumption (Lovitt et al. 1988, Girbal and Soucaille 1994, Payot et al. 1998, Willquist et al. 2011). In order to recycle the excess of NADH and keep the catabolic metabolism to proceed, carbon and electron flux must be diverted to alternative NADH-oxidizing reactions such as lactate, ethanol and alanine synthesis. Although this scenario is based on the data gathered mainly from *Ca. saccharolyticus* fermentations (Bielen et al. 2013a), according to the information in the literature (i.e. high NADH/NAD^+ triggering

alternative pathway and inhibition of GAPDH by NADH) it seems plausible that this explanation can be extended to other thermophilic H_2 producers. Thus, under high $[H_2]_{aq}$ one microorganism can sustain growth if it is capable of maintaining NADH/NAD^+ at homeostatic levels by switching the metabolism. As a consequence of the changes in the fermentation profile, acetate, H_2 and ATP yields are reduced.

Based on this assumption, tolerance towards H_2 is a function of the degree of inhibition that high NADH/NAD^+ exerts towards GAPDH and of the mechanisms to maintain redox homeostasis. A lower sensibility of GAPDH towards NADH allows a higher intracellular accumulation of NADH under H_2ase-inhibiting conditions without negatively affecting the glycolytic flux. For example, a mutant *Th. thermohydrosulfuricus* strain, whose GAPDH showed higher tolerance towards NADH compared to the wild-type variant, was found to be more tolerant towards H_2 than the wild-type strain (Lovitt et al. 1988). Similarly, *Ca. saccharolyticus* possesses a fairly tolerant GAPDH compared to other related organisms (e.g. *Th. thermohydrosulfuricus* and *Cl. acetobutylicum*) allowing to support a high glycolytic flux also at elevated $[H_2]_{aq}$ (Willquist et al. 2011). The activation of alternative pathways to dispose excess of NADH under H_2ase-inhibiting conditions has been found to be under transcriptional and allosteric control. In fermentative organisms the expression of a large number of genes in the central carbon metabolism, including those involved in the disposal of reducing equivalents, is under the control of the Rex regulator (Ravcheev et al. 2012). This transcriptional factor acts as a negative regulator and responds to the intracellular redox state, in particular to the NADH/NAD^+ ratio: at the low NADH/NAD^+ ratio, the Rex protein binds to the target sites repressing the transcription of controlled genes, while the increase of NADH concentration results in the dissociation of Rex from DNA and derepression of its target genes (Brekasis and Paget 2003, Pagels et al. 2010, Pei et al. 2011, Ravcheev et al. 2012). For example, Rex was found to control the redox-dependent solventogenic shift in *Cl. acetobutylicum* (Wietzke and Bahl 2012) and end-product synthesis in *Staphylococcus aureus* (Pagels et al. 2010). In *Caldicellulosiruptor* spp. the Rex regulon includes genes involved in the fermentative metabolism (i.e. FeFe-H_2ase, NiFe-H_2ase, alcohol dehydrogenase, pyruvate:ferredoxin oxidoreductase, oxaloacetate decarboxylase) whose upregulation was observed in *Ca. saccharolyticus* under high P_{H2} suggesting a Rex-mediated transcriptional regulation (Bielen et al. 2013b). Also in *Thermotoga* spp. the expression of FeFe-H_2ase and alcohol dehydrogenase is under the control of Rex (Ravcheev et al. 2012). Alternatively, the synthesis of metabolites aiding to control the excess of NADH can be regulated at post-transcriptional level. Both in *Ca. saccharolyticus* and *T. maritima* the upstream region of gene encoding for lactate dehydrogenase does not show a Rex binding motif. However, several effectors such as nicotinamide adenine dinucleotides, adenosine phosphates, fructose-1,6-biphosphate, orto- and pyrophosphate are known to modulate the activity of lactate dehydrogenase (Palmfeldt et al. 2004, van Niel et al. 2004a, Cao

et al. 2010a, Willquist and van Niel 2010). Thus, lactate production can be affected by the intracellular level of these compounds that in turn depends on the physiological state of the cell.

Combining experimental data and knowledge of genome content, some additional genetic biomarkers for H_2 tolerance have been proposed. Shaw et al. (2009) suggested a correlation between the presence in the genome of *hfs* cluster encoding for a Fd-dependent FeFe-H_2ase containing a PAS/PAC sensory domain and the ability of some anaerobic fermentative bacteria to grow under a H_2 atmosphere (P_{H2}= 185 kPa). However, no further in-depth analysis was performed to investigate the molecular basis of this observation. Interestingly, some thermophilic H_2-producing species found to withstand high concentration of H_2 encode a PFL. PFL, like PFOR, catalyzes the conversion of pyruvate to acetyl-CoA. Despite the similar $\Delta G^{0'}$ for reactions catalyzed by PFOR and PFL (-19.2 and -16.3 kJ/mol, respectively) (Thauer et al. 1977), an efficient reoxidation of Fd_{red}, generally by H_2 production, is necessary for maximum activity of PFOR, whereas non-oxidative pyruvate dissimilation does not depend on the availability of oxidized cofactors. As a consequence, even at high H_2 concentration the thermodynamics of the reaction catalyzed by PFL does not change, whereas the reaction catalyzed by PFOR is expected to be less favorable due to the increased $\Delta G'$ of the ferredoxin oxidation. Therefore, such a branched pyruvate node can serve as a safety valve relieving the cell from the burden of ferredoxin reoxidation in unfavorable conditions and thus avoiding a decrease in the metabolic flux at this catabolic step. For example, the growth rate of *C. celer* and *Cl. thermocellum* is minimally affected at high H_2 concentration (Rydzak et al. 2011, Carere et al. 2014, Ciranna et al. 2014a). In these conditions the catabolic flux and the acetyl-CoA supply are maintained unaltered by partially shifting the carbon and electron flow at the pyruvate node towards the reaction catalyzed by PFL. However, in absence of a formate hydrogen lyase (FHL) that further converts formate to H_2 and CO_2 the H_2 yield decreases.

1.2.4. Factors affecting thermophilic H_2 production

A deep knowledge of the microbial physiology is crucial to develop a robust biotechnological process. The cellular metabolism and the external cellular environment are intimately related and ultimately the phenotype of a cell is the result of the interaction between these two components. The extracellular variables that characterize the environment, the "envirome" (Lidén 2001), influence the intracellular redox and energetic state of the cell and in turn affect the performance of a bioprocess. Consequently, the parameters pertaining to the bioprocess must be optimized in order to maximize the output. Conventionally, most optimization studies employed a so-called one-factor-at-a-time technique. Although simple, this approach is time-consuming and ignores the interaction between the tested variables. Therefore, statistical optimization

methods, such as response surface methodology, are employed to overcome these disadvantages allowing for optimization of multiple variables with minimum number of experiments. While statistical methods have recently been widely employed to optimize biohydrogen production process under mesophilic conditions (Nath and Das 2011), only few studies are available for thermophiles (O-Thong et al. 2008b, Cao et al. 2010b, Mamimin et al. 2012). Fermentative H_2 production is a very complex process influenced by many factors (Wang and Wan 2009). Here, some key parameters are discussed with particular emphasis on their role in thermophilic H_2 production.

pH

Fermentative H_2 production has been observed in a wide range of pH (Wang and Wan 2009, Kivistö et al. 2013a). The disagreement on the optimal pH to employ for H_2 production can partly be attributed to the different experimental setups (non-pH-controlled vs. pH-controlled fermentations) employed in different studies. Certainly, the optimal value needs to be studied case by case as it depends on the metabolic and physiological properties of the microorganism under investigation. Moreover, the ideal pH for cell growth may not be the same as the one for maximal H_2 production (Ciranna et al. 2014a). Surprisingly, studies on the effect of pH on thermophilic H_2 production are scarce. In fact, the role of pH in the fermentative metabolism of the model organisms for thermophilic H_2 production has never been thoroughly investigated. Slightly acidic pH (6-7) is suggested to be optimal for thermophilic H_2 production (Pawar and Van Niel 2013).

The culture pH can profoundly affect intracellular redox and energetic state of several fermentative organisms. However, the effect of pH on the carbon and electron flux and thus on end-product yields is organism-specific. For example, in several fermentative cultures grown under controlled conditions a pH-dependent metabolic shift was observed as culture pH was increased, resulting in a reduction of H_2 production and an increase of formate accumulation (Temudo et al. 2007, Lee and Rittmann 2009, Liu et al. 2011, Ciranna et al. 2014a). In contrast, the metabolism of several strains of *Cl. acetobutylicum* has been reported to switch from acidogenesis and H_2 production to solventogenesis when the pH was lower than 5 (Jones and Woods 1986). In *Cl. cellulolyticum* acidic pH shifted the carbon and electron flow from H_2/acetate to ethanol/lactate synthesis, and caused bottlenecks in the catabolic metabolism with subsequent decrease of substrate consumption and overflow metabolism at the pyruvate node (Desvaux et al. 2001). In addition, low pH enhances the detrimental effect of accumulation of organic acids on cellular metabolism by favoring the formation of their undissociated form (Jones and Woods 1986, Van Ginkel and Logan 2005a). High concentrations of undissociated organic acids also contribute to the shift towards solventogenic metabolism in clostridia (Jones and Woods 1986).

In general, the maintenance of an optimal culture pH can be simply achieved by addition of buffering or caustic agents to the medium. However, on an industrial scale their use is not advisable. In fact, life cycle and techno-economic analyses foresee a negative contribution of these two medium components to overall costs and environmental impact of the process (Ochs et al. 2010, Ljunggren et al. 2011b). In addition at high concentrations they can increase the osmotic pressure in the medium inhibiting bacterial growth and H_2 production (Willquist et al. 2009). This outlook makes the control of pH in the H_2 production process very challenging, especially if acid-pretreated feedstocks (e.g. lignocellulosic hydrolysates) are used as substrate.

Temperature

The effect of the temperature on the performance of hydrogenogenesis can be assessed by testing the same inoculum on a broad range of temperatures or by comparing data from the literature for mesophilic and thermophilic pure cultures. The first approach shows that thermophilic conditions favor higher substrate-to-H_2 conversion yields and specific H_2 production rate (Yokoyama et al. 2007a, Gavala et al. 2006, Lin et al. 2008, Lin et al. 2012). This could be explained by the more favorable thermodynamics of the reactions (see section 1.2.2), the lower production of microbial mass (Gavala et al. 2006) as well as the decreased microbial diversity that allows for selection of more efficient H_2 producers (e.g. *Thermoanaerobacterium* spp., *Thermoanaerobacter* spp., *Clostridium* spp. and *Caloramator* spp.) and more limited production of by-products (Shin et al. 2004, Karadag and Puhakka 2010). The second approach draws similar conclusions. A comprehensive comparison of the data available in literature from studies of pure cultures shows that thermophiles achieve higher conversion yields and specific H_2 production rates, whereas mesophilic condition favors volumetric H_2 production rates (Rittmann and Herwig 2012). Again, favorable thermodynamics, limited production of by-products and low biomass production might be responsible for the temperature-dependent differences in the performance (Chou et al. 2008, Verhaart et al. 2010). When dealing with thermophilic pure cultures it is important to identify the optimal temperature for growth which usually corresponds to the one for maximal H_2 yield and production rate (van Niel et al. 2004b, O-Thong et al. 2008a, Ren et al. 2008).

Only few studies have been carried out to investigate how changes in temperature impact the whole cellular system. Proteomic studies on two model thermophilic H_2 producers show that the comprehensive protein response to increased temperature involves components of the central carbon metabolism and thus relevant for the fermentative ability of these organisms. In *Cal. subterraneus* subsp. *tengcongensis* a lower abundance of enzymes with redox-regulation capacity (including the NADH-dependent FeFe-H_2ase) as well as of proteins related to respiration of proton, Fe(III), sulfur and sulfate was observed as the temperature increased, whereas several proteins partaking in glycolysis and the related energy production were found to be significantly

upregulated (Wang et al. 2007, Chen et al. 2013). The dramatic decline of growth rate in *Cal. subterraneus* subsp. *tengcongensis* at a temperature just 5 °C above the optimal one (75 °C) (Xue et al. 2001) can be explained with the lower abundance of the proteins dealing with disposal of electrons and oxidative stress. In *T. maritima* the level of proteins in the carbohydrate and energy metabolism were found to be sensitive to environmental thermal stress (Wang et al. 2012). In particular a number of upregulated proteins were located downstream of glyceraldehyde-3-phosphate. Therefore, central carbohydrate metabolism pathways of *T. maritima* are likely activated at higher temperature.

End-product inhibition and osmolality

During anaerobic fermentation several metabolites (gases, organic acids and alcohols) are produced and accumulated in the system. At high concentrations products of the fermentative metabolism can exert an inhibitory effect. As a result, growth inhibition, incomplete substrate conversion and changes in metabolite production profile can occur due to impaired cellular metabolism (Nicolaou et al. 2010). Both H_2 and CO_2 have been found to negatively affect H_2 production and microbial growth (Van Niel et al. 2003, Willquist et al. 2009). Even though both should be removed from the reactor, the effect of CO_2 has been generally overlooked, while H_2 usually receives more attention. As previously discussed, the concentration of H_2 in the system has a huge impact on H_2 production, mainly influencing the thermodynamics of the biological reactions. The prediction of a critical H_2 concentration that causes inhibition of H_2 production (yield and productivity) and/or microbial growth has been a challenging task due to the multiple variables involved. The critical $[H_2]_{aq}$ for *Ca. saccharolyticus* grown on glucose has been observed at 2.2 mM of dissolved H_2 (Ljunggren et al. 2011a). However, it is likely that the critical $[H_2]_{aq}$ will depend on the organism, its physiological state and the external conditions.

High dissolved H_2 concentrations should be avoided in order to efficiently operate a fermentative H_2 production process. This goal can be achieved by increasing the mass transfer rate of H_2 from the liquid to the gas phase (Ljunggren et al. 2011a). Gas sparging methods have been tested at lab-scale to reduce the H_2 concentration. The use of nitrogen (N_2) has been extensively studied and sparging the fermentation vessel with this gas guarantees low H_2 concentration in the system and high H_2 yields (Schröder et al. 1994, Soboh et al. 2004, Nguyen et al. 2010, Zeidan and van Niel 2010, Ngo et al. 2011, Willquist et al. 2011, Ciranna et al. 2012, Kivistö et al. 2013b, Carere et al. 2014, Ciranna et al. 2014a). However, at commercial scale the use of N_2 would not be practical because it is inert and thus difficult to separate from the effluent gas stream (Van Groenestijn et al. 2002). CO_2 would be a convenient option because it is a product of the fermentative process and it is easier to separate. In contrast to N_2, CO_2 is known to negatively affect the microbial metabolism and to modify the composition of the

culture medium (Dixon and Kell 1989, Willquist et al. 2009). Methane has also been evaluated as a stripping gas showing to improve H_2 yield (Liu et al. 2006, Pawar et al. 2013). As CO_2, it can be produced *in situ* by hybrid biohydrogen/biogas process and similarly to N_2 it does not affect the metabolism of fermentative organisms. In alternative to gas sparging the mass transfer rate can be increased by improving the reactor design (Peintner et al. 2010, Sonnleitner et al. 2012) (see section 1.2.5). The removal of H_2 could also be achieved by operating under reduced pressure (Mandal et al. 2006, Junghare et al. 2012, Sonnleitner et al. 2012) or by employing selective membranes for separation of H_2 (Lu et al. 2007b). Both methods have been shown to improve the performance of the process (Mandal et al. 2006, Lee et al. 2007), however they might be challenging to implement at industrial scale due to practical and economic factors.

Soluble metabolic products (organic acids and alcohols) inhibit H_2 production and microbial growth at high concentrations. In addition, the presence of these molecules at subinhibitory concentrations can alter the distribution of the carbon and electron flow resulting in stimulatory or inhibitory effects towards the production of the desired end-product (Grupe and Gottschalk 1992, He et al. 2009, Rydzak et al. 2011, Tang et al. 2012, Ciranna et al. 2014b). Organic acids can uncouple bacterial growth in two ways: the nonpolar undissociated form can freely diffuse into the cell and release protons in the cytoplasm with consequent disruption of the proton motive force and increase of cellular maintenance energy (Jones and Woods 1986), whereas the polar dissociated form contributes to the increase of the ionic strength in the solution which at high level can inhibit microbial growth and cause cell lysis (Van Niel et al. 2003). The ratio between the two forms is dependent on the culture pH and the dissociation constant (pK_a) of the organic acid: as the pH decreases below the pK_a value the fraction of undissociated acids increases above 50 % of the total and vice versa. The threshold concentration of undissociated carboxylic acids that significantly inhibits H_2 production (and triggers solventogenesis) or cell growth was found in the 20-50 mM range (Wang and Wang 1984, Grupe and Gottschalk 1992, Van Ginkel and Logan 2005a, Akutsu et al. 2009).

However, in few thermophilic H_2 producers (e.g. *Ca. saccharolyticus* and *C. celer*) the high osmolality exerted by the dissociated fraction was the main culprit for the inhibition (Van Niel et al. 2003, Willquist et al. 2009, Ciranna et al. 2014b). This indicates that these species are not particularly osmotolerant and that under optimal growth conditions (pH close to neutrality and low substrate concentration) the critical ionic strength (0.4-0.6 M of solutes) is reached before the level of undissociated acids becomes inhibiting. In contrast, thermophilic H_2 producers isolated from marine environments (e.g. *T. maritima* and *P. furiosus*) display a higher osmotolerance requiring about 0.5 M NaCl for optimal growth thanks to their ability to synthetize

compatible solutes (Martins and Santos 1995, Martins et al. 1996). Pawar and van Niel (2013) identified in the tolerance to high osmotic stress, exerted by high substrate/by-product concentrations, one of the desirable features that an ideal H_2-producing microorganism should possess. However, the osmosensitivity of some thermophilic H_2 producers imposes to operate at low substrate loading rates resulting in reduced H_2 productivity. At industrial scale this would translate in the need for bigger reactors to maintain a profitable H_2 output with consequent increase of water and energy demand (Ljunggren et al. 2011b). Osmosensitive cultures can not be sparged with CO_2 for the removal of H_2 due to the need to add extra base to neutralize medium acidification with consequent increase of the osmotic pressure (Willquist et al. 2009). In addition, high osmolality has shown to increase the sensitivity to dissolved H_2 (Ljunggren et al. 2011a). Hence, the osmotolerance is an important factor in the development of a robust and cost-effective H_2 production process.

Ethanol can also be accumulated during dark fermentation as a means to balance the intracellular redox state. Solvents exert inhibition on the bacterial cell by damaging and denaturing biological molecules and imparting biophysical changes to cell membranes (Nicolaou et al. 2010). Thermophiles are generally less tolerant towards ethanol than mesophiles (Lynd 1989, Burdette et al. 2002, Georgieva et al. 2007). In fact, most of them tolerate only 1-2 % v/v and only few natural strains and adapted strains can stand ethanol concentration up to 8 % v/v (Sudha Rani and Seenayya 1999, Burdette et al. 2002, Georgieva et al. 2007). However, under conditions favoring H_2 synthesis ethanol is synthetized in low quantities and thus its inhibition should not raise particular concerns for the stability of the process. Moreover, in *Cl. thermocellum* and *C. celer* small concentrations of ethanol have been found to slightly improve H_2 yield (Rydzak et al. 2011, Ciranna et al. 2014b). A similar behavior observed in *Th. ethanolicus* 39E grown on pyruvate with increasing concentrations of ethanol was justified by the possible use of ethanol as an electron donor (reverse electron flow), which in turn increased the NADH available for H_2 production, rather than with feedback inhibition of the aldehyde/alcohol dehydrogenase activity (Lovitt et al. 1988).

Medium composition

In order to support microbial growth at high rate during dark fermentation the medium needs to be supplied not only with carbon and energy sources but also with other macronutrients (i.e. nitrogen, phosphorus, sulfur) and micronutrients (e.g. iron, nickel, vitamins, etc.). Medium composition can play a relevant role in the optimization of the H_2 production process, but can also contribute substantially to the costs associated with microbial growth and waste processing (Ochs et al. 2010, Ljunggren et al. 2011b). For this reason it would be important to minimize the medium components while maintaining or even improving the performance of the process.

Thus far, most of the studies on thermophilic H_2 producers have been performed employing complex media often containing excess of nutrients (Pawar and Van Niel 2013). Yeast extract (YE) and peptone are often supplemented to the growth medium as organic nitrogen sources providing amino acids but also buffering capacity, reducing agents and chelators for metal ions. However, their use is not cost-effective at industrial scale (Ljunggren et al. 2011b), thus optimal H_2 producers should be able to grow on minimal media (Pawar and Van Niel 2013). Nevertheless, YE and/or peptone can generally stimulate microbial growth and H_2 production (van Niel et al. 2002, Kongjan et al. 2009, Ciranna et al. 2011, Martinez-Porqueras et al. 2013, Maru et al. 2013), but in few cases rich medium has been found to cause metabolic imbalances leading synthesis of other end-products (Willquist and van Niel 2012) or growth inhibition (Payot et al. 1998). In addition, these compounds often serve as a carbon and energy source possibly making the study of metabolic fluxes inaccurate. Ammonium salts were also tested as inorganic nitrogen sources, but their use resulted in decreased H_2 production (Kalil et al. 2008, O-Thong et al. 2008a, Martinez-Porqueras et al. 2013). The use of urea as primary low-cost nitrogen source is intriguing because it eliminates the need for base addition for neutralizing protons released during ammonium assimilation (Shaw et al. 2012).

Sulfur and iron are important micronutrients for H_2 fermentation as they are constituents of H_2ases and ferredoxin (Vignais and Billoud 2007). Since thermophilic H_2 producers are extremely sensitive to oxygen, the growth medium is always supplemented with sulfur-based reducing agents (e.g. L-cysteine, sodium sulfide, sodium dithionite) to scavenge oxygen. *Caldicellulosiruptor* spp. and *Cl. thermocellum* can utilize both organic and inorganic sulfur sources (Zeidan 2011, Kridelbaugh et al. 2013). However, the effects of different sulfur sources and their assimilation on H_2 production have been poorly characterized. In contrast, the effect of iron in hydrogenogenesis is better understood. H_2ases contain a high number of iron atoms (roughly 12 Fe per H_2ase in prokaryotes) (Roessler and Lien 1984), thus under iron limitation H_2ases are either not synthesized or synthesized but not in a functional form (Junelles et al. 1988). Consequently, in these conditions the reducing equivalents can not be disposed via proton reduction and therefore the fermentative metabolism shifts from H_2 and acetate production to the synthesis of alternative reduced end-products (e.g. lactate, ethanol, butanol, etc.) (Junelles et al. 1988, Peguin and Soucaille 1995, Ciranna et al. 2011). The iron content (both as Fe^{2+} and Fe^{3+}) in the biomass of *E. coli* is estimated to be 14.4 $\mu mol/g_{CDW}$ (Feist et al. 2007) and thus an iron concentration in the 25-40 μM range should suffice for fermentative growth (van Niel et al. 2011). However, several studies report much higher concentration for optimal H_2 production (up to 6 mM) (Lee et al. 2001, Zhang and Shen 2006, Ciranna et al. 2011), indicating that availability and/or cellular uptake of the iron in the medium might be lower than expected. Other micronutrients (trace elements and vitamins) are likely to have a physiological role in

the metabolism of thermophilic H_2 producers. Limitation or excess of micronutrients can influence pathways in the central carbon metabolism affecting the fermentation profile (Kivistö et al. 2011, Lin et al. 2011). Thus far, information on the effect of trace elements and vitamins on thermophilic H_2 production is however scarce.

1.2.5. Challenges and future perspectives

Improving process performances: yield, productivity and cell growth

Although studies in the literature clearly indicate the superior ability of thermophiles to produce H_2 at high yields, when considering the overall performances of fermentation at high temperatures two matters worth of consideration are the most significant limitations for industrial-scale application: low cell growth and low volumetric H_2 productivity. Biomass formation in thermophilic H_2 producers is reported to be from few folds up to more than one order of magnitude lower than most mesophilic bacteria (Chou et al. 2008). Among the reasons proposed for the limited cell growth in thermophiles there are the sensitivity to high osmotic pressure (Van Niel et al. 2003), the occurrence of metabolic shift at high H_2 concentration towards less efficient metabolic routes (Verhaart et al. 2010, Willquist et al. 2010) and the preference of more favorable electron acceptors such as elemental sulfur (Schicho et al. 1993, Schut et al. 2007). A direct consequence of the poor cell growth is the unsatisfactory volumetric H_2 productivity to establish an economically feasible H_2 production process. Low productivity has a severe impact on the capital and maintenance costs, since bigger sized reactors would be needed to achieve an acceptable energy output over time (Ljunggren and Zacchi 2010, Ljunggren et al. 2011b). Ideally, the process should generate H_2 at high yield and rate. However, a trade-off between H_2 productivity and yield is generally observed mainly due to mass transfer limitations at high production rates resulting in increased $[H_2]_{aq}$ and consequent metabolic shift (Willquist et al. 2010). This imposes to choose either a high yield-oriented process or a high productivity-oriented process depending on process-specific aspects, e.g. the cost of the feedstock and the organism(s) (Pawar and Van Niel 2013).

The reactor configuration certainly impacts the performance of thermophilic fermentation and a smart reactor design would help overcoming the aforementioned shortcomings by improving cell densities and liquid-to-gas mass transfer rates. Most of the studies on thermophilic H_2 production, especially from pure cultures, are carried out in continuous stirred-tank reactor (CSTR) which allows to operate under well-controlled laboratory conditions but is not ideal for efficient biomass retention (Ren et al. 2011, Pawar and Van Niel 2013). Reactors that favor the formation of granules or biofilms can maintain higher biomass concentrations at lower hydraulic retention time (HTR) and thus should be preferred to increase the productivity (Ren et al. 2011). In few

studies the H_2 production from thermophilic species was investigated using alternative reactor setups such as trickling bed reactors (Van Groenestijn et al. 2009, Peintner et al. 2010), fluidized bed reactors (Peintner et al. 2010), gas-lift reactors (Kanai et al. 2005), upflow anaerobic sludge blanket reactors (O-Thong et al. 2008c) in order to improve the biomass retention and to enhance the mass transfer properties. Most notably *Th. thermosaccharolyticum* PSU-2 immobilized on heat-pretreated methanogenic granules achieved a volumetric H_2 production rate of 152 mmol H_2/l/h, the highest reported to date from a pure thermophilic culture (O-Thong et al. 2008c). Accumulation of fermentative metabolites at high concentrations inhibits biomass formation and shifts the metabolism away from H_2 production. Alternative methods to the inconvenient N_2 sparging for decreasing the effect of H_2 build-up in the reactor were tested with *Ca. saccharolyticus* by inducing bubble formation and by reducing the pressure in the bioreactor (Sonnleitner et al. 2012). Cell-recycling and dialysis methods were successfully used to remove liquid end-products allowing an increase of cell mass in *Th. brockii* and *P. furiosus* (Holst et al. 1997).

Promising results for improving the performance of the H_2 production process have been obtained exploiting metabolically complementary microbial populations by designing *de novo* co-cultures. Besides increasing yield and production rate, and favoring biomass formation (Zeidan and Van Niel 2009, Zeidan et al. 2010), this approach should particularly benefit the conversion of complex feedstocks by expanding the metabolic capabilities of the culture and overcoming substrate utilization bottlenecks. For example, *Cl. thermocellum* is a very efficient cellulose-degrader but struggles to completely ferment all the soluble product of the hydrolysis (e.g. cellobiose and glucose) whose accumulation in the medium inhibits its cellulolytic activity (Johnson et al. 1982). Co-culturing *Cl. thermocellum* with non-cellulolytic H_2 producers able to efficiently metabolize the product of cellulose breakdown led to improved cellulose degradation and H_2 yield compared to the monoculture of *Cl. thermocellum* (Liu et al. 2008, Geng et al. 2010, Li and Liu 2012).

Further developments of the dark fermentative H_2 production technology by implementing the aforementioned strategies might help to enhance the performances of the process. Yet, its efficiency is constrained within the metabolic boundaries of fermentative microbes that limit the yield to 4 mol H_2/mol hexose, only 33 % of the H_2 released from complete hexose oxidation. In order to overcome this limitation and make biohydrogen production economically feasible on a commercial scale, dark fermentation should be coupled with a second step allowing energy recovery from by-products in the effluent. This energy can be retrieved in form of H_2 via photofermentation and electrohydrogenesis (Hallenbeck 2009, Hallenbeck and Ghosh 2012). The photofermentation by purple photosynthetic bacteria uses sunlight along with organic substrate for H_2 production. However, this method faces challenges including low light

conversion efficiency and costly photobioreactor systems. The electrohydrogenesis relies on a small electric current to convert the organic acids to H_2 in microbial electrolysis cells (MEC). The technology has been studied extensively during the recent years and promising development has been obtained, although more research is needed to prove its practical potential. Another attractive choice for improving the conversion efficiency is to generate methane (CH_4) from the organic acids produced during dark fermentation by anaerobic digestion, a methanogenic reliable and industrially established process (Hallenbeck 2009, Hallenbeck and Ghosh 2012). The product of this two-stage process is a H_2/CH_4 mixture (hythane) that burns considerably cleaner than methane alone.

Genetic engineering and systems biology

A deep knowledge of the central carbon metabolism and of the bacterial physiology is crucial to design an efficient fermentative H_2 production process. In order to achieve this goal, powerful tools (e.g. genetic engineering, omics technologies, genome-scale models) are nowadays available to better understand the biological systems as a whole and possibly overcome physiological barriers. Genetic engineering offers attractive solutions to improve fermentative H_2 production: i) bypassing metabolic bottlenecks in H_2-producing bacteria by eliminating competing pathways (e.g. lactate dehydrogenase and/or alcohol/aldehyde dehydrogenase) (Yoshida et al. 2006, Li et al. 2010, Cha et al. 2013) or by enhancing H_2-evolving reactions (e.g. H_2ase) (Von Abendroth et al. 2008, Jo et al. 2010, Zhao et al. 2010), albeit constrained within the 4 mol H_2/mol glucose; ii) introducing novel synthetic pathway that elevate the yields above the theoretical limits (Chittibabu et al. 2006); iii) design more robust and tolerant strains towards inhibitors (e.g. osmotic pressure, end-products, inhibitors in feedstocks) (Nicolaou et al. 2010). Additionally, perturbation studies based on gene knock-out are an important instrument for investigating the physiological role of a specific enzyme (Shaw et al. 2009, Kanai et al. 2011, Lipscomb et al. 2011). Most of the studies in this area have been carried out in mesophilic enterobacteria due to their ease of genetic manipulation, whereas strict anaerobic mesophiles and thermophiles are not as genetically tractable. Culture handling under anaerobic conditions, scarcity of selection markers, construction of adequate shuttle vectors and presence of restriction modification (RM)-systems preventing foreign DNA to be uptaken by the cell are the main limitations for establishing reliable and efficient transformation protocols (Taylor et al. 2011, Bosma et al. 2013, Pawar and Van Niel 2013). In recent years, some genetic tools have been developed for few thermophilic H_2 producers, although the majority of these organisms are still not genetically accessible. The most important progresses for enhancing transformation efficiencies in fermentative thermophiles include methods for methylation of foreign DNA (Chung et al. 2012, Guss et al. 2012), removal of RM-systems (Chung et al. 2013a), screening of genetically tractable strains (Bosma et al.

2013), construction of specific shuttle vectors (Farkas et al. 2011, Han et al. 2012, Olson and Lynd 2012, Chung et al. 2013b), identification or development of naturally competent strains (Shaw et al. 2010, Lipscomb et al. 2011), alternative techniques to electroporation (Lin et al. 2010), development of markerless gene deletion methods based on counter-selection systems (Tripathi et al. 2010, Argyros et al. 2011, Shaw et al. 2011) and construction of a thermotargetron for gene deletion based on mobile group II introns (Mohr et al. 2013).

New possibilities for modeling metabolic networks arise from combining high-throughput data sets derived from proteome, transcriptome and metabolome analysis with the information obtained from a growing number of annotated genomes (Joyce and Palsson 2006, Feist et al. 2009). The well-established construction of genome-scale metabolic models (Thiele and Palsson 2010) allows an *in silico* investigation of a biological system by employing computational tools such as flux balance analysis (FBA) (Orth et al. 2010). The use of genome-scale metabolic models to test metabolic hypothesis and to guide genetic engineering strategies has been successfully used to boost the production of several bioproducts (Lee et al. 2005, Oberhardt et al. 2009, Santala et al. 2011, Yim et al. 2011). In recent years, genome-scale metabolic models have been also constructed for thermophilic H_2 producers (Zhang et al. 2009, Roberts et al. 2010, Munro et al. 2011, Zeidan 2011, Nogales et al. 2012). These models can be employed to test *in silico* the introduction of non-native pathways in existing H_2 producers to increase the production yield beyond the theoretical limits. For example, metabolizing glucose through the oxidative branch of the PPP and disposing all the reducing equivalents through H_2 production should yield 8 mol H_2/mol glucose, two moles of ATP and one mole of acetate (Hallenbeck and Benemann 2002, De Vrije et al. 2007). Such pathway has never been observed in H_2-producing organisms. *In silico* redesign of glycerol and glucose metabolisms in *T. maritima* showed that, when H_2 production was selected as an optimization criterion, H_2 yield could be increased beyond the theoretical limits if carbon flux is funneled through the oxidative PPP and if NADPH and NADH/Fd pools are connected by introducing non-native reactions (Nogales et al. 2012). The introduction in *E. coli* of similar pathways minimally improved H_2 yield possibly due to thermodynamic limitations, which can not be predicted by the model, and natural optimization of growth rate instead of H_2 production by microorganisms (Veit et al. 2008, Kim et al. 2011b). Testing *in vivo* this as well as other model-driven hypotheses in thermophilic H_2 producers remains a challenge due to the lack of well-established genetic tools for most of them. Nevertheless, genome-scale metabolic models can be useful for other studies (Oberhardt et al. 2009), including investigation of nutritional requirements and medium optimization (Zeidan 2011, Willquist and van Niel 2012, Kridelbaugh et al. 2013).

1.3. THE CALORAMATOR GENUS

The genus *Caloramator* is a recently discovered taxonomic group with an increasing number of members. Although poorly characterized compared to other closely related genera (e.g. *Clostridium*, *Caldicellulosiruptor*, *Thermoanaerobacter*, etc.), the few emerging studies indicate that *Caloramator* species might have potential for a variety of biotechnological applications thanks to their interesting metabolic traits. This chapter aims to give a comprehensive review on this genus with particular relevance to the potentially exploitable properties for biotechnological applications.

1.3.1. Taxonomy and phylogeny

In 1994 several species belonging to the genus *Clostridium* were reclassified in new genera (Collins et al. 1994). Among these, *Cl. fervidus* was relocated in the new genus *Caloramator*. Since then, several new species belonging to the "heat lover" genus have been isolated and characterized. The genus *Thermobrachium* with its type and only species *Tb. celere* was initially classified as a separate taxonomic group due to the evolutionary distance from *Clostridium fervidus*, at the time the only member of the *Caloramator* genus (Wiegel 2009). Only later with the description of other *Caloramator* species it was revealed that *Thermobrachium celere* was within the radiation of the genus *Caloramator* given the close evolutionary distance to some *Caloramator* species such as *C. indicus*. Consequently, *Tb. celere* was assigned to the genus *Caloramator* as *C. celer* (Baena and Patel 2009).

The 16S rDNA analysis places the members of the genus *Caloramator* as a cohesive cluster in the family *Clostridiaceae*, order *Clostridiales*, class *Clostridia*, phylum *Firmicutes*. Presently, 11 species have been isolated and described (Figure 5, Table 9) and few more strains have not been assigned to any species. Additionally, more than 40 sequences of 16S rDNA have been taxonomically assigned to the genus *Caloramator* (http://www.ncbi.nlm.nih.gov/nuccore). According to the phylogenetic analysis (Figure 5), three subclusters can be identified within the *Caloramator* genus. However, given the poor characterization of this genus, it is yet unknown whether species within one group share common genotypic and phenotypic traits.

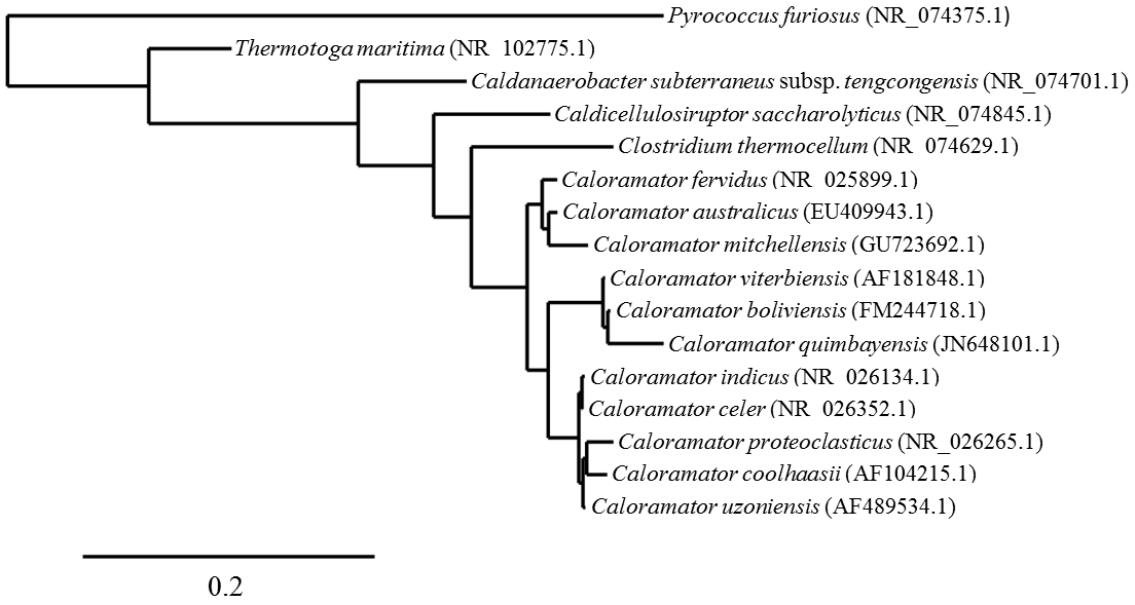

Figure 5. Phylogenetic dendrogram based on 16S rDNA gene sequence of the Caloramator *genus and their relation with other fermentative thermophilic microorganisms.*

1.3.2. Isolation and cultivation

Caloramator spp. were isolated from terrestrial sources mainly from hot springs or non-volcanically heated water, but few species (*C. proteoclasticus* and some strains of *C. celer*) were found in mesophilic environments (e.g. mesophilic reactors, river and lake sediments) (Table 9). Given the anaerobic nature of these bacteria, isolation, enrichment and cultivation procedures were performed using traditional anaerobic techniques such as modified Hungate technique (Ljungdahl and Wiegel 1986) or roll-tube technique (Hungate 1950). Enrichment of the environmental samples was carried out in media supplemented with YE and/or peptone along with or without other carbon sources. Only *C. boliviensis* was enriched in a minimal medium with wheat straw as carbon source (Crespo et al. 2012a). Interestingly, *C. australicus* and *C. mitchellensis* were isolated after a screening procedure in microplates for identification of microorganisms with dissimilatory iron(III)-reducing and vanadium(V)-reducing activity, respectively (Ogg and Patel 2009, Ogg and Patel 2011). Serial dilution in liquid medium and streaking on solid medium were the main methods for isolation of pure cultures with the exception of *C. boliviensis* isolated in a biphasic medium (Crespo et al. 2012a).

Table 9. Phenotypic traits of established members of the genus Caloramator. Caloramator uzoniensis *is not included in the table since no published description is available.*

	Caloramator fervidus	***Caloramator celer***	***Caloramator indicus***	***Caloramator proteoclasticus***	***Caloramator coolhaasii***	***Caloramator quimbayensis***	***Caloramator boliviensis***	***Caloramator viterbiensis***	***Caloramator australicus***	***Caloramator mitchellensis***
Isolation source	Hot spring, New Zealand	Hot spring, New Zealand	Non-volcanically heated waters, India	Mesophilic granular methanogenic sludge	Anaerobic thermophilic granular sludge	Hot spring, Colombia	Hot spring, Bolivia	Hot spring, Italy	Microbial mat, Australia	Bore-water sample, Australia
Morphology & size (µm)	Rods, 2-2.5×0.65-0.75	Rods and branched filaments, 1.5-14×0.5-1.2	Rods and filaments, 10-100×0.6-0.8	Slightly curved rods, 2.4-4.0×0.4	Rods and filaments 2-40×0.5-0.7	Straight to slightly curved rods, 2.5×0.9	Straight to slightly curved rods, 2.5×0.5	Straight to slightly curved rods 2.0-3.0×0.4-0.6	Slightly curved rods, 2.5-4.2×0.8-1.0	Slightly curved rods, 1.5-3.5×0.4-0.8
Gram stain	Negative	Positive	Negative	Negative	Negative	Positive	Variable	Positive	Positive	Negative
G+C (mol %)	39	31.3	25.6	31	31.7	32.6	32	32	32.8	38.4
Spores	+	-	-	+	-	+	+	-	-	-
Doubling time (min)	45	10	20	30	60	120	84	168	32	34
Motility	+	+	-	+	-	+	+	-	+	+
Temp. range (°C)	37-80	43-75	37-65	30-68	37-65	37-55	45-65	33-64	45-70	37-60
Optimal temp (°C)	68	66	60-65	55	50-55	50	60	58	60	55
pH range	5.5-9.0	5.4-9.5	6.2-9.2	6.0-9.5	6.0-8.0	6.0-8.0	5.5-8.0	5.0-7.8	6.0-9.0	6.0-9.0
Optimal pH	7.0-7.5	8.2	7.5-8.1	7.0-7.5	7.0-7.5	7.0	6.5-7.0	6.0-6.5	7.0	7.0
YE/peptone required	+	+	+	+	+	+	-	ND	+	+
Growth on amino acids[a]	Ser	ND	ND	Glu, met, arg, his, thr, leu, val, gly	Glu, asp, ala, arg, met	Ala, arg, glu, ile, val	ND	Ser, glu, thr, leu, met, asp, his, val	Thr	Ser
Growth on substrates[b]	Glc, mal, xyl, sta, xln, man, pyr	Glc, fru, gal, mal, suc	Glc, fru, sta, amy, dex, amp, clb, lac, man, suc	Glc, fru, sta, clb, man	Glc, fru, gal, suc, mal, rib, xyl, sta, celb, man	Glu, fru, ara, mal, mnl, man, rib, suc, sta	Xyl, clb, glu, ara, suc, lac, mal, fru, gal, man, gly, xln, cmc	Glu, fru, suc, clb, lac, gal, sta, man, gly	Glu, fru, gal, xyl, mal, suc, raf, man, clb, cel, sta, amp, xln, pyr, gly	Glu, fru, xyl, mal, suc, raf, clb, rib, pyr
End-products from glucose fermentation[c]	Ace, etOH, lac, bVFA, CO_2, H_2	Ace, etOH, for, but, CO_2, H_2	Ace, etOH, lac, CO_2, H_2	Ace, etOH, lac, for, CO_2, H_2	Ace, lac, CO_2, H_2	Ace, for, etOH, lac, CO_2, H_2	Ace, etOH, lac, pro, CO_2, H_2	Ace, etOH, CO_2, H_2	Ace, etOH, CO_2, H_2	Ace, etOH, CO_2, H_2
Dissimilatory metal reduction	-	ND	ND	V(V), Fe(III), Mn(IV)	V(V), Fe(III)	-	ND	Fe(III), Mn(IV)	V(V), Fe(III), Mn(IV)	V(V)
References	Patel et al. 1987	Engle et al. 1996	Chrisostomos et al. 1996	Tarlera et al. 1997	Plugge et al. 2000	Rubiano-Labrador et al. 2013	Crespo et al. 2012a	Seyfried et al. 2002	Ogg and Patel 2009	Ogg and Patel 2011

ND: not determined; +, yes; -, no

a) Amino acid abbreviations: ala, alanine; arg, arginine; asp, asparagine; glu, glutamate; gly, glycine; his, histidine; ile, isoleucine; leu, leucine; met, methionine; thr, threonine; ser, serine; val, valine

b) Substrate abbreviations: ara, arabinose; cel, cellulose; clb, cellobiose; dex, dextrin; fru, fructose; gal, galactose; glc, glucose; gly, glycerol; lac, lactose; mal, maltose; man, mannose; mnl, mannitol; pyr, pyruvate; raf, raffinose; rib, ribose; sta, starch; suc, sucrose; xln, xylan; xyl, xylose

c) Metabolite abbreviations: ace, acetate; but, butyrate; bVFA, branched volatile fatty acids; etOH, ethanol; for, formate; lac, lactate; pro, propionate

1.3.3. Morphology and physiology

Cells of the genus *Caloramator* are straight to slightly curved rods or filaments measuring 2-100 μm × 0.4-0.8 μm, and occur singly, or in pairs (Table 9). In *C. celer* cells with a branched morphology were also observed as well as L-form cells in stationary phase (Engle et al. 1996). Gram staining is variable depending on the species and growth phase (Table 9). However, ultrathin sections of cells reveal a multilayered, complex, thick cell wall with an external S-layer similar to the one of Gram-positive cell walls. Therefore, *Caloramator* spp. are considered to be Gram-positive (Wiegel 1981). Heat-resistant endospore formation occurs in some but not all the species (Table 9). If present, spores are spherical and located terminally or subterminally. Motility is also variable depending on the species. Some have sluggish or tumbling mobility thanks to mono- or peritrichous flagella, whereas others are not motile (Table 9). *Caloramator* spp. are strictly anaerobic and (moderately) thermophilic chemoorganotrophs that grow at temperatures ranging from 30 to 80 °C, with an optimum between 50 and 68 °C. The pH range for growth is between 5 and 9.5 and the optimum pH for growth is between 6 and 8.2 (Table 9). Two *Caloramator* spp., *C. celer* and *C. indicus*, can be considered alkalithermophiles being their optimal growth conditions 66 °C and pH 8.2, and 63 °C and pH 8.1, respectively (Table 9). Only two species, *C. boliviensis* and *C. quimbayensis*, show a generation time higher than 1 hour (Table 9). Therefore, *Caloramator* spp. can be generally considered fast-growing bacteria. In particular, *C. celer* was reported to grow with the shortest doubling time of 10 minutes, making this bacterium the fastest among the anaerobic organisms.

1.3.4. Metabolism and nutritional requirements

Most of the members of the genus are able to metabolize a wide range of substrates including carbohydrates, amino acids/proteins and sugar alcohols. The carbohydrates utilized to support growth in *Caloramator* spp. are glucose, starch, amylose, maltose, xylan, mannose, galactose, raffinose, fructose, ribose, xylose, mannose, sucrose, cellulose, cellobiose, carboxymethylcellulose, amylopectin, lactose, galactose and dextrin (Table 9). The use of polymeric components of lignocellulose was reported only for some species. In particular, cellulolytic activity was observed only in *C. boliviensis* and *C. australicus*, and xylanolytic activity in *C. boliviensis*, *C. australicus* and *C. fervidus* (Table 9). Also glycerol was metabolized only by few species, namely *C. boliviensis* and *C. viterbiensis*. Most of the species require YE and/or tryptone/peptone for growth, suggesting auxotrophy for some amino acids or need for trace components present in these compounds. Indeed, the reconstruction of amino acid biosynthetic pathways from the genome sequences of *C. celer* and *C. australicus* revealed that these species are auxotrophic for several amino acids due to incomplete biosynthetic routes

(our unpublished results). However, *C. boliviensis* requires YE only for growth on glycerol (Crespo et al. 2012a) and *C. coolhaasii* can grow without YE on glutamate (Plugge et al. 2000), provided in both cases that the medium is supplemented with vitamins. YE and/or tryptone/peptone alone as well as some free amino acids can support limited growth in absence of other fermentable substrates in all species.

Members of the genus *Caloramator* possess a fermentative metabolism. According to the genome analysis of four members of the genus (*C. celer*, *C. australicus*, *C. boliviensis* and *C. proteoclasticus* ALD01) it is likely that the EMP pathway is the main glycolytic route for the conversion of carbohydrates to pyruvate (Crespo 2012b, our unpublished results). The meta-analysis of the sequenced *Caloramator* spp. shows that the oxidative PPP and the ED pathway are lacking or largely incomplete, suggesting that they are not involved in sugar metabolism. However, the reductive branch of the PPP is present. Pyruvate is then converted to several metabolic products such as acetate, ethanol, lactate, propionate, butyrate, formate, branched volatile fatty acids, CO_2 and H_2, with species-dependent fermentation profile (Table 9). Additionally, *C. boliviensis* and *C. viterbiensis* can produce 1,3-propanediol from degradation of glycerol. Detailed studies on the sugar metabolism of *Caloramator* genus were carried out only in two species: *C. celer* and *C. boliviensis*. The results showed that these two species possess a very flexible metabolism. (Crespo 2012b, Ciranna et al. 2014a). Despite the branched metabolism of *C. boliviensis*, changes in the cultivation conditions promoted homoacetogenic, homolactic, homoethanolic metabolism as well as lactate-based butyrate metabolism (Crespo 2012b). *C. celer* shifted its metabolism in response to several fermentation parameters to simultaneously control redox state and efficiently harvest energy from substrate even under unfavorable conditions (Ciranna et al. 2014a).

As previously mentioned YE, tryptone, peptone and some free amino acids can support growth in *Caloramator* spp. without the addition of other fermentable substrates (Table 9). This suggests that amino acid fermentation is a common metabolic trait within the *Caloramator* genus. Amino acid fermentation was studied in *C. proteoclasticus* and *C. coolhaasii* (Tarlera et al. 1997, Tarlera and Stams 1999, Plugge et al. 2000, Plugge et al. 2001, Plugge and Stams 2002). These two species can metabolize glutamate mainly to acetate, H_2, CO_2, NH_4^+, as well as other by-products (i.e. formate, alanine, ethanol and propionate). Measurements of enzymatic activities and nuclear magnetic resonance (NMR) analysis showed that acetate formation and consequent production of ATP by substrate level phosphorylation from glutamate occurs via β-methylaspartate pathway and not via hydroxyglutarate pathway (Tarlera and Stams 1999, Plugge et al. 2001). Degradation of proteins, glutamate and other amino acids to acetate is severely affected by P_{H2} resulting in decreased growth rate and production of reduced metabolites. Indeed, co-cultures with hydrogenothrophic methanogens relieved the inhibition with consequent increase of consumption rates and acetate production (Tarlera and Stams

1999, Plugge and Stams 2002). Among the two species, only *C. proteoclasticus* showed the ability to degrade some amino acids (e.g. valine and leucine) via Stickland reaction using glycine as electron acceptor (Tarlera and Stams 1999). This reaction couples the oxidation of one amino acid with the reduction of another yielding organic acids, CO_2 and NH_4^+ (but not H_2) and variable amount of ATP depending on the amino acids pair and their ratio (Nisman 1954). Degradation of other free amino acids was reported for *Caloramator* spp. with variable type and amount of end-products (Table 9), although their catabolic routes remain unknown.

Some members of the *Caloramator* genus are capable of dissimilatory reduction of various metals such as iron(III), manganese(IV), vanadium(V), arsenate (V) (Table 9) (Ledbetter et al. 2007, Ogg and Patel 2009, Ogg and Patel 2011). However, these species are not obligate dissimilatory metal-reducing microorganisms because they do not rely on metals as the only respiratory electron acceptors for growth. Moreover, this metabolic activity was observed on a limited number of electron donors.

1.3.5. Biotechnological applications

Despite the limited number of studies on *Caloramator* spp., few technological applications have been envisioned for these species based on their interesting metabolic features. *Caloramator* spp. can metabolize a wide array of substrates, including polymeric carbohydrates, hexose and pentose sugars, sugar alcohols and proteins (Table 9), and therefore they can be employed in bioconversion of renewable feedstocks or waste streams to added-value products. Growth on lignocellulosic hydrolysates was tested only in *C. boliviensis* and in a newly isolated *C. proteoclasticus* strain (ALD01) (Crespo et al. 2012c, Elkins et al. 2012). Interestingly, *C. proteoclasticus* ALD01 not only readily grew in the presence of biomass hydrolysates, but was able to convert furan and aromatic aldehydes, commonly known to act as inhibitors, to less-toxic alcohols (Elkins et al. 2012). In addition, it rapidly reduced 15 mM furfural to furfuryl alcohol during growth on glucose. This suggests the ability of this microorganism to grow on lignocellulosic feedstock in presence of pretreatment inhibitors. If this detoxification activity is a shared trait of the genus, the use of *Caloramator* spp. alone or in co-culture with other organisms will enable the use of non-detoxified hydrolysates. The proteolytic activity reported for the wild-type strain of *C. proteoclasticus* and *C. celer* could be exploited in treatments of protein-rich industrial wastewaters with high chemical oxygen demand (COD) (e.g. from slaughterhouses, tanneries and dairy industries). For this purpose, *C. proteoclasticus* was investigated using proteins as substrates (Tarlera and Stams 1999). *C. proteoclasticus* was able to convert casein and gelatin to a variety of volatile fatty acids and H_2. When *C. proteoclasticus* was cultured with a hydrogenotrophic thermophilic methanogen faster degradation of proteins and higher

specific activity of the proteases were observed, highlighting the inhibiting effect of H_2 and the importance of synthrophic growth in amino acid fermentation.

Table 10. The potential biotechnological applications envisioned for Caloramator *spp.*

Application	Feedstock	Species/Strain	Reference
Detoxification of lignocellulosic hydrolysates	Pretreated lignocellulosic biomass	*C. proteoclasticus* ALD01	Elkins et al. 2012
Wastewater treatment	Protein-rich industrial wastewaters	Proteolytic species (*C. proteoclasticus*, *C. celer*)	Tarlera and Stams 1999 Engle et al. 1996
Biohydrogen production	Pretreated lignocellulosic biomass	All species	Table 9
Bioethanol production	Pretreated lignocellulosic biomass	All species (except *C. coolhaasii*)	Table 9
1,3-propanediol production	Glycerol wastes	*C. boliviensis*, *C. viterbiensis*	Crespo et al. 2012a Seyfried et al. 2002
Bioelectricity in MFC	Organic substrates	Species with dissimilatory metal-reducing activity (e.g. *C. australicus*)	Fu et al. 2013
Removal of toxic metal	Contaminated water systems	*Caloramator* YeAs	Ledbetter et al. 2007
Synthesis of nanomaterials (e.g. β-realgar)		*Caloramator* YeAs	Ledbetter et al. 2007

The ability of these microorganisms to produce H_2 and ethanol as final products of the fermentation has raised the interest for exploring their potential in biofuels production. *C. celer* and a closely related strain (T42) have been investigated for their potential to produce H_2. *C. celer* converted glucose to H_2 at relatively high yields up to 88 % of the theoretical maximum (i.e. 4 mol H_2/mol glucose) (Ciranna et al. 2012), whereas strain T42 reached only 1.06 mol H_2/mol glucose (Niu et al. 2006). In addition, several strains phylogenetically related the *Caloramator* genus have been often found in thermophilic H_2-producing mixed consortia enriched from environmental samples (Yokoyama et al. 2007b, Koskinen et al. 2008b, Hniman et al. 2011a, Hniman et al. 2011b, Kongjan et al. 2011). *C. boliviensis* and a strain closely related to *C. viterbiensis* enriched from an Icelandic hot spring converted both glucose and xylose to ethanol with a yield of 94 and 75 %, and 66 and 37 % of the theoretical maximum (i.e. 0.51 g ethanol/g sugar), respectively (Orlygsson et al. 2010, Crespo 2012b). *C. boliviensis* produced ethanol also from a renewable feedstock (pentose-rich sugarcane bagasse hydrolysate) under continuous operation mode with a yield comparable to that of other ethanologenic thermophiles (Crespo et al. 2012c).

If produced from cheap substrates, the organic acids produced during anaerobic fermentation (e.g. acetate, formate, lactate, butyrate) can serve as platform chemicals. But perhaps, one of the most interesting chemical for the polymer industry is 1,3-propanediol. The production of this compound from glycerol has been widely

investigated in mesophilic organisms, but reports available for thermophilic production are scarce (Wittlich et al. 2001, Arbige 2004). *C. viterbiensis* and *C. boliviensis* are capable of converting glycerol to 1,3-propanediol and their potential for the production of this compound should be explored more in depth.

Another potential application for *Caloramator* spp. is the generation of bioelectricity in microbial fuel cells (MFC). Two strains related to *Caloramator* spp. were involved in electricity generation in a thermophilic sludge-inoculated MFC. Further investigations confirmed the exoelectrogenic activity of *C. australicus* that couples the oxidation of organic substrates to the reduction of the electrode (Fu et al. 2013). Also other *Caloramator* spp. could be able to generate electricity given their ability to reduce various metals. In addition, this metabolic activity could be exploited for the biogenic mineralization of metals with implications in several scientific fields including nanotechnology for the production of novel minerals and nanomaterials, and bioremediation for the immobilization and removal of toxic metals from contaminated water systems (Ledbetter et al. 2007).

2. HYPOTHESES AND OBJECTIVES

In the quest for developing a robust and efficient dark fermentative process for H_2 production at industrial scale, the organism(s) selected to carry out the bioconversion is crucial. The search for this organism can be pursued by following two strategies: bioprospecting new H_2 producers with enhanced characteristics or improving the known species by directed evolution strategies and/or genetic engineering. It is likely that the development of the ideal organism for a successful fermentative H_2 production process will require a confluence of both strategies.

In this study, a novel bacterium, *Caloramator celer* (former *Thermobrachium celere*), was evaluated for its potential to produce H_2 from organic substrates. Koskinen et al. (2008b) showed that a thermophilic mixed culture producing H_2 at high yield was dominated by bacterial strains closely affiliated with *C. celer*, suggesting the high potential for fermentative H_2 evolution by this species. *C. celer* is an anaerobic alkalithermophilic bacterium with an optimal growth temperature of 67 °C, an optimal $pH^{67°C}$ of 8.2 and a doubling time reported as low as 10 minutes (Engle et al. 1996). A microorganism with such characteristics could possess some advantages for a hydrogenogenic process: i) the great selective pressure provided by the optimal growth conditions would allow to outcompete H_2-consuming microorganism in an open (non-sterile) bioprocess system; ii) the remarkably high growth rate would translate in high volumetric production rates.

The general goal of this study was to investigate the fermentative metabolism of *C. celer* by determining the relationship between fermentation conditions, physiological state, genome content, gene expression, metabolic fluxes and end-product yields. At the beginning of the study it was hypothesized that:

- *C. celer* was an efficient thermophilic H_2 producer and could produce H_2 at high yield and rates;
- fermentation conditions and medium composition could influence physiological state, metabolic fluxes and in turn end-product yields;
- high concentration of substrate and end-products could inhibit growth and H_2 production;
- gene expression patterns affected distribution of carbon and electron flux;
- knowledge of the genome content allowed a more systematic investigation of the metabolic potential of *C. celer* for H_2 production and provided valuable

information for interpretation of experimental results and directing experimental design.

Based on these hypotheses, the objectives of the thesis were:

- to assess the performance of *C. celer* as an H_2 producer and optimize the fermentation conditions and medium composition (Paper **I**, **II**, **IV**);
- to prove that physiological state, metabolic fluxes and end-product yields are affected by fermentation conditions such as medium composition (Paper **I**, **II**), pH (Paper **I**, **IV**), accumulation of H_2 in the system (Paper **I**, **II**, **IV**), presence of soluble end-product in the medium during fermentation (Paper **III**);
- to evaluate the tolerance of *C. celer* towards high concentration of substrate and end-products (Paper **III**, **IV**) and to identify the mechanism of inhibition of the most abundant soluble metabolites (i.e. acetate) in the fermentation medium (Paper **III**);
- to elucidate the relationship between level of gene expression and distribution of metabolic fluxes under perturbed conditions (i.e. addition of subinhibitory concentration of soluble metabolites) (Paper **III**);
- to obtain the genome sequence of *C. celer* (Paper **V**) and to utilize genomic data for transcriptional analysis (Paper **III**), for construction of a stoichiometric model employed in metabolic flux analysis (MFA) (Paper **IV**) and for comparative genomic analysis (Paper **VI**).

3. SUMMARY OF MATERIALS AND METHODS

In this section a summary of materials and methodologies used in this study is given. For more detailed information refer to the original publications (Paper **I-VI)**.

3.1. Strain, medium and culture handling

Caloramator celer strain JW/YL-NZ35, former *Thermobrachium celere* (equivalent to DSMZ 8682 and ATCC 700318), was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *C. celer* was cultivated in a modified ATCC 2072 medium (www.lgcstandards-atcc.org/~/media/4866DB6C29C44938B4B62542D2152259.ashx). The basic version of the medium contained (per liter): KH_2PO_4 0.64 g; Na_2HPO_4 1.65 g; KCl 1 g; $(NH_4)_2SO_4$ 0.5 g; NH_4Cl 0.5 g; $MgCl_2 \cdot 6H_2O$ 0.1 g; $CaCl_2 \cdot 6H_2O$ 0.11 g; cystein-HCl 0.13 g; $Na_2S \cdot 9H_2O$ 0.13 g; resazurin 0.001 g; trace element solution 10 ml; vitamin solution 10 ml; yeast extract 2 g. Modifications to the basal medium composition were made depending on the experimental setup (Table 11). Glucose was used as substrate in all experiments. Medium, stock solutions and bacterial cultures were maintained and handled in anaerobic conditions using the modified Hungate technique (Ljungdahl and Wiegel 1986).

Table 11. Modifications made to the basal medium and glucose concentrations in the study.

Paper	Modifications	Glucose concentration
I		25 mM, 50mM
II	KH_2PO_4 1.24; Na_2HPO_4 5.79; $FeSO_4 \cdot 7H_2O$ 0.2 g; tryptone 2 g	55 mM
III	KH_2PO_4 1.24; Na_2HPO_4 5.79; $FeSO_4 \cdot 7H_2O$ 0.2 g; tryptone 2 g	0-300 mM
IV	KH_2PO_4 0.75 g; $Na_2HPO_4 \cdot 2H_2O$ 1.53 g; $FeSO_4 \cdot 7H_2O$ 0.2 g; tryptone 2 g; cystein-HCl 0.2 g; $Na_2S \cdot 9H_2O$ omitted	27.5 mM, 55 mM

3.2. Experimental setup

The experiments in this study were performed in batch mode in 120 ml serum bottles with culture volumes from 10 to 90 ml under non-controlled conditions (Paper **I-III**) or in a 3-liter continuous stirred-tank reactor (CSTR) with a working volume of 1 l under controlled conditions (Paper **IV**) (Table 12). All the cultures were maintained at 67 °C. All the experimental conditions were conducted at least in biological duplicates and included negative controls omitting glucose.

Table 12. Fermentation setups employed during this study.

Paper	Fermentation type	Fermentation vessel	Controlled conditions	Initial culture volume
I	Batch	Serum bottles	No	10-90 ml
II	Batch	Serum bottles	No[a]	20 ml
III	Batch	Serum bottles	No	20 ml, 200 ml[b]
IV	Batch	CSTR	Yes	1000 ml

a) one 3M NaOH pulse for manual pH adjustment

b) split into 20 ml samples when early exponential phase was reached

3.2.1. Process optimization

In order to optimize the H_2 production process during glucose fermentation by the alkalithermophile *C. celer*, the influence of several chemo-physical parameters and nutritional requirements on growth, H_2 production and fermentative metabolism was investigated in this study (Table 13). In addition, data from batch cultures in CSTR was employed to investigate the effect of two parameters, pH and P_{H2}, on fermentative metabolism and distribution of carbon and electron flux to end-products (Paper **IV**).

Table 13. Parameters optimized in this study and the objective of the optimization.

Parameter		Range	Objective	Paper
pH	Initial value	6.2-9.2[a]	H_2	I
	Constant value	5.5-8.0[b]	H_2, growth, metabolic flux analysis	IV
Buffer	Concentration[c]	0-50 mM	H_2	I
	Type[d]	Phosphate	H_2, growth	II
		Carbonate	H_2, growth	
		HEPES	H_2, growth	
H_2 partial pressure	Gas-to-liquid ratio	0.75-12	H_2	I
	Headspace flushing with N_2		H_2, growth	II
	Continuous N_2 sparging	0 or 6 l/h	H_2, growth, metabolic flux analysis	IV
Yeast extract		0-5 g/l	H_2, substrate consumption	I
Tryptone		0-5 g/l	H_2, substrate consumption	I
$FeSO_4 \cdot 7H_2O$		0-200 mg/l	H_2	I
Substrate	Glucose	0-300 mM	H_2, growth	I, III

a) measured at 67 °C

b) measured at room temperature

c) phosphate buffer

d) at 50 mM

3.2.2. Inhibitory effects of substrate and soluble end-products

The degree of inhibition exerted by the three main soluble metabolic products (acetate, formate and ethanol) and by the substrate load on the growth, H_2 production and fermentative metabolism of *C. celer* was investigated in this study (Table 14) (Paper **III**). The tolerance towards the tested compounds and the mechanism of inhibition were assessed by analyzing the kinetics of growth and H_2 production (Table 18). In addition, the effect of the addition of subinhibitory concentrations of acetate, formate and ethanol

on the fermentative metabolism and the expression profile of key genes in pyruvate catabolism and end-product formation was studied (see section 3.2.3) (Paper **III**).

Table 14. Experiments performed in this study to investigate the inhibition of substrate and soluble metabolic products.

Inhibiting agent		Range	Addition	Kinetic parameter analyzed	Objective	Paper
Acetate	Na-acetate	0-350 mM	BI	μ_{max}, Q_{H2}	Inhibition kinetics	III
	Na-acetate	0-425 mM	AI	Q_{H2}	Mechanism of inhibition	III
	K-acetate	0-360 mM	AI	Q_{H2}	Mechanism of inhibition	III
	Na-acetate at pH 8	0-425 mM	AI	Q_{H2}	Mechanism of inhibition	III
	Na-acetate at pH 6	0-425 mM	AI	Q_{H2}	Mechanism of inhibition	III
	Na-acetate	80 mM	BI	/	Fermentative metabolism	III
Formate	Na-formate	0-350 mM	BI	μ_{max}, Q_{H2}	Inhibition kinetics	III
	Na-formate	40 mM	BI	/	Fermentative metabolism	III
Ethanol		0-350 mM	BI	μ_{max}, Q_{H2}	Inhibition kinetics	III
		40 mM	BI	/	Fermentative metabolism	III
Ionic strength	NaCl	0-425 mM	AI	Q_{H2}	Mechanism of inhibition	III
	KCl	0-360 mM	AI	Q_{H2}	Mechanism of inhibition	III
Substrate	Glucose	0-300 mM	BI	μ_{max}, Q_{H2}	Inhibition kinetics	III

AI, after inoculation; BI, before inoculation; μ_{max}, maximum specific growth rate (h^{-1}); Q_{H2}: volumetric H_2 production rate (mmol H_2/l/h) measured during exponential phase

3.2.3. Transcriptional analysis

The expression level of 12 genes involved in the pyruvate catabolism and end product synthesis was quantified by designing 14 sets of primers (including two housekeeping genes) with amplification efficiency between 90 and 105 % (Table 15). The total RNA was isolated and purified from cell cultures in late exponential phase of growth and retrotranscribed to cDNA. The cDNA levels were analyzed by SYBR® Green-based real-time quantitative PCR (qPCR) (Paper **III**).

3.2.4. Genome analysis

The isolated and purified genomic DNA of *C. celer* was sequenced with Illumina HiSeq 2000 (at the Institute for Molecular Medicine Finland) to get paired-end reads from short (~250 bp) and long (~3 kbp) fragment libraries, as well as with 454 sequencing (at the University of Helsinki) to get longer single-end reads. The draft genome was analyzed to elucidate transport and utilization of carbohydrates, production of fermentative end-products, electron shuttling, transcriptional regulation and energy conservation systems (Paper **V**, **VI**). A stoichiometric model used for metabolic flux analysis (MFA) and a scenario of generation and consumption of reducing equivalents in *C. celer* were proposed based on these findings and experimental results (Paper **IV**).

Table 15. Primers used to investigate the effect of subinhibitory concentrations of soluble metabolic products on the expression of 12 genes putatively involved in pyruvate catabolism and end-product formation.

Primers		Gene target	Putative enzyme	Gene locus
pfl_f	GATATGATGTTTCAAGACCAGCAAAG	*pfl*	Pyruvate formate lyase	TCEL_00503
pfl_r	ACTTACTCTACCTAGTGACATAGCAGCAC			
pflae_f	GGAGGCGGAGTTACACTTTCAG	*pfl-ae*	Pyruvate formate lyase activating enzyme	TCEL_00502
pflae_r	TGTGAATTCCTTCTTCCTTACACCT			
por1_f	ACCACTTGATCTTCCAAACCACTAC	*porA*	Pyruvate:ferredoxin oxidoreductase	TCEL_02203[a]
por1_r	TTCAGCAGCAACTTCAAGAATTACTC			
por2_f	GTTTACTCAAATACAGGTGGTCAATCA	*por*	Pyruvate:ferredoxin oxidoreductase	TCEL_01566
por2_r	GCTCATTGCTATCATTCCAAGGT			
pta_f	GGTTGCTGAATTAAAGGCTCCA	*pta*	Phosphotransacetylase	TCEL_01822
pta_r	GCTCCTGCTAATCTTTGAACTAACTTG			
acka_f	CAGGAGTTCTTGGTATTTCAGGTGTA	*ack*	Acetate kinase	TCEL_01823
acka_r	AATGGAATACATCAAGTGCTAGTTGTG			
hyd1_f	TGCAGCAGACCTTACAATAATGGA	*hydA*	NADH-dependent FeFe hydrogenase	TCEL_01277[a]
hyd1_r	ACAGCAGCTTGTCATAAGTGGAAG			
hyd2_f	GACGCTGTGAGACAATGTGTAATG	*hydA*	NADH-dependent FeFe hydrogenase	TCEL_00581[a]
hyd2_r	AGGCAGGTCCTACAACTGTTTCA			
mbhl_f	GACAGGCGTTAGAAAACCTTCCA	*mbhL*	Ferredoxin-dependent NiFe hydrogenase	TCEL_00200[a]
mbhl_r	TGGTGCTTCGTGTCTTGCTGTA			
mbhj_f	GTATCACTAGGATCATGCCCAAGA	*mbhJ*	Ferredoxin-dependent NiFe hydrogenase	TCEL_00188[a]
mbhj_r	GCCATTATAGCTTCTGGTTTTGGA			
adhe_f	CAGTTAAAGCTGGAGCACCAAAG	*adhE*	Aldehyde/alcohol dehydrogenase	TCEL_01373
adhe_r	GAAGAGTAGGCAGCCTTTACCATTC			
bdh_f	GAACAGAGGTTACAAGGGCATCAG	*bdh*	Alcohol dehydrogenase	TCEL_00064
bdh_r	CATTCCTGTTTCAGCAACAACTTC			
polc_f	TGGATGGAGTAACATCTGCAACA	*polC*	DNA polymerase	TCEL_01861[b]
polc_r	CATAGCTTCAGGGAATGCTTGA			
reca_f	GAAATGGGAGATGCTTTTGTAGGA	*recA*	Bacterial DNA recombination protein	TCEL_01881[b]
reca_r	TTCAGGACTACCAAACATAACACCAA			

a) Transcript level of one gene was used to assess the expression of operons coding for putative multimeric enzymes.
b) Housekeeping gene

3.3. Analytical methods and calculations

Cultures of *C. celer* were regularly sampled and analyzed for monitoring bacterial growth, pH, substrate consumption, gaseous and liquid metabolite formation (Paper **I-IV**), expression of key genes in pyruvate catabolism and end-product formation (Paper **III**), intracellular nicotinamide adenine dinucleotides (NAD^+ and NADH) and intracellular adenosine triphosphate (ATP) (Paper **IV**). The genome of *C. celer* was sequenced, assembled, annotated, manually curated and analyzed (Paper **V**, **VI**). Methods of MFA were employed to calculate the unknown intracellular fluxes in *C. celer* in response to changes in culture pH and P_{H2} (Paper **IV**). Noncompetitive equations of growth inhibition and a four parameter logistic (dose-response) equation were used to estimate the kinetics of the inhibition exerted by the substrate, inorganic salts and soluble metabolites on bacterial growth and H_2 production rates (Paper **III**). The $\Delta\Delta C_T$ method was used to calculate the relative gene expression and *p*-value was determined to assess the statistical significance (Paper **III**). All analytic methods and calculations used in this study are listed in Table 16, Table 17 and Table 18.

Table 16. Summary of the analysis performed on the bacterial cultures during the study.

Analysis	Method	Paper	References
Gaseous compounds; *H_2, CO_2*	Gas chromatography	I-IV	
Off-gas volume	Gas-tight syringe	I-III	
	Displacement of $NaHCO_3$-saturated solution	IV	
Soluble compounds; *glucose, acetate, ethanol, formate, butyrate, pyruvate*	High-performance liquid chromatography	I-IV	
pH	pH electrode	I-IV	
Biomass;			
Optical density	Spectrophotometer (600 or 620 nm)	I-IV	
Cell dry weight	Centrifugation + drying + weighting	I-III	
	Filtration + drying + weighting	IV	Willquist and van Niel 2010
Transcriptional analysis	RNA isolation and purification	III	
	cDNA synthesis	III	
	Evaluation of primer efficiency	III	
	Real-time quantitative PCR	III	
NAD(H)	Cyclic assay	IV	Willquist et al. 2011
ATP	Bioluminescence-based assay	IV	Willquist et al. 2011

Table 17. Summary of the methods, databases and softwares employed for genome analysis, transcriptional analysis, metabolic flux analysis and inhibition kinetics experiments.

Analysis	Method/Software/Database	Paper	References/Manufacturers
Genome sequencing;		V	
Sequencing	Illumina pair-end, 454 technologies		
Assembly	MIRA		Chevreux et al. 1999
Contig extension and scaffolding	SSPACE		Boetzer et al. 2011
Automatic annotation	RAST server		Aziz et al. 2008
	IMG-ER platform		Markowitz et al. 2009
Manual annotation	NCBI-Blast		Altschul et al. 1997
Phylogenetic analysis;		VI	
Multiple sequence alignment	MUSCLE		Edgar 2004
Alignment curation	Gblocks		Castresana 2000
Tree construction	PhyML		Guindon and Gascuel 2003
Tree visualization	Treedyn		Anisimova and Gascuel 2006
Genome analysis[a]	Integrated Microbial Genomes (IMG)	III,IV,VI	Markowitz et al. 2009
	Clusters of Orthologous Groups (COG)		Tatusov et al. 2000
	KEGG Orthology (KO)		Kanehisa et al. 2008
	TIGRFAMs		Haft et al. 2003
	Transporter Classification Database		Saier Jr. et al. 2009
	MUSCLE		Edgar 2004
	Genome Explorer		Mironov et al. 2000
	Genome context analysis		
	Literature searches		
Data fitting of inhibition kinetics	Origin 8	III	Originlab (USA)
Primer design	Primer express	III	Invitrogen (USA)
Statistical analysis of transcriptional analysis	Data assist	III	Invitrogen (USA)
Metabolic flux analysis	MATLAB	IV	The MathWorks (USA)

a) employed for identification of key genes in pyruvate catabolism and product formation (Paper **III**), construction of a genome-based stoichiometric model (Paper **IV**), and comparative genomic analysis (Paper **VI**)

Table 18. Most significant equations employed for calculations in the study. For explanation of the parameters refer to the original publications.

Eq.	Formula	Objective	Paper	References
6	$r = r_{max} * (1-C/C_{crit})^n * S/(S+K_s)$	Inhibition kinetics of substrate	III	Han and Levenspiel 1988
7	$r = r_{max}/(1+C/K_c)^n$	Inhibition kinetics of soluble metabolites	III	Tang et al. 2012
8	$r_{norm} = a+\{(b-a)/[1+(C/C_{50})^n]\}$	Inhibition kinetics of soluble metabolites	III	DeLean et al. 1978
9	$r = r_{max} * (1-C/C_{crit})^n$	Mechanism of inhibition by acetate	III	Van Niel et al. 2003
10	$\Delta\Delta C_T$	Gene expression level	III	Pfaffl 2001
11	$r = (dC/dt) * X$	Specific rates for MFA	IV	Stephanopoulos et al. 1998
12	$V_c = -G_c^* \times G_m \times V_m$	Calculation of intracellular fluxes	IV	Stephanopoulos et al. 1998
13	$Y_{X/ATP} = [bio]/(2*[ace]+1*[eth])$[a]	Yield of biomass per ATP	IV	Stephanopoulos et al. 1998
14	$Y_{ATP/S} = (2*[ace]+1*[eth])/[glu]$[a]	ATP yields	IV	Stephanopoulos et al. 1998

a) assumes that *C. celer* uses a PEP-dependent phosphotransferase system (PTS) for glucose uptake

4. SUMMARY OF RESULTS AND DISCUSSION

4.1. Characterization of fermentation

Caloramator celer metabolizes glucose via mixed acid fermentation as generally reported for H_2-producing microorganisms. The main products of the glucose fermentation were H_2, CO_2, acetate, ethanol and formate in accordance with the description of Engle et al. (1996). Trace amounts of butyrate (generally below 1 mM) were also detected depending on the growth conditions. Production of butyrate was not originally reported by Engle et al. (1996). In most of the experiments, acetate resulted to be the major soluble metabolite, whereas synthesis of ethanol and formate varied drastically depending on the fermentation conditions. Occasionally, pyruvate was detected in the supernatant suggesting overflow metabolism at the pyruvate branch point (Paper **III**). Besides H_2 and CO_2, no other gases (e.g. H_2S) were identified.

C. celer requires at least 1 g/l of YE to sustain growth (Engle et al. 1996), indicating auxotrophy for some amino acids. Additionally, *C. celer* utilizes YE and tryptone both as carbon and energy sources without the addition of other fermentable substrates. Fermentation of YE and/or tryptone yielded H_2, CO_2, acetate, ethanol and traces of butyrate, but no formate. Parallel fermentations with and without addition of glucose showed that under optimal conditions for H_2 production the contribution of YE and tryptone to the final accumulation of H_2 and acetate was about 15-20 % and 10-15 %, respectively (Paper **I**). This observation is in accordance with an estimation performed by NMR technique showing that in *T. neapolitana* the contribution of YE and tryptone to the overall acetate production accounted for about 12 % (d´Ippolito et al. 2010). Since these values are not negligible, the contribution of YE and proteic hydrolysates must be taken into account in the calculation of the end-product yields.

4.2. Optimization of medium composition and fermentation conditions for H_2 production

The effect of several parameters on growth and fermentation of *C. celer* was investigated in order to identify the optimal conditions for H_2 production. The optimal conditions that maximize H_2 production (either as H_2 yield or H_2 productivity) and/or bacterial growth for each of the tested factors are described in this section and summarized in Table 19.

Table 19. Values of the parameters tested in this study (normal text) resulting in the highest H_2 yield, H_2 productivity, H_2 accumulation, specific growth rate and biomass formation (bold text). The values of the parameters are reported as a range when the significance of the results (based on the standard deviation) did not allow to identify a single optimal value. Each parameter was investigated in independent experiments under different conditions (as indicated in the footnotes) and consequently a large variation in fermentation performances (bold text) can be observed.

Parameter	Parameter value for the highest				
	H_2 yield (mol H_2/mol glu)	**H_2 productivity (mmol H_2/l/h)**	**H_2 accumulation (mmol H_2/l)**	**Specific growth rate (h^{-1})**	**Biomass formation (g_{CDW}/l)**
Glucose (mM)	50 **(2.95)**[c]	100 **(22)**[c]	50 **(101)**[c]	25-50 **(0.53)**[c]	25-100 **(0.51-0.54)**[c]
Phosphate buffer (mM)	12.5 **(2.67)**[c]	ND	50 **(50)**[c]	ND	ND
Buffering agent	Phosphate **(2.84)**[c]	HEPES-Carbonate **(31)**[c]	HEPES **(116)**[c]	Carbonate **(2.15)**[c]	Phosphate-Carbonate **(0.44)**[c]
Yeast extract (g/l)	2 **(3.16)**[c]	ND	2 **(114)**[a,c]	ND	ND
Tryptone (g/l)	0 **(3.16)**[c]	ND	2 **(114)**[a,c]	ND	ND
$FeSO_4 \cdot 7H_2O$ (mg/l)	200 **(3.08)**	ND	200 **(112)**	ND	ND
Soluble end-product (mM):					
Acetate	20-150 **(≈2.90)**[c]	0 **(39)**[c]	40-80 **(≈108)**[c]	0-40 **(0.70-0.78)**[c]	0-80 **(0.51-0.56)**[c]
Formate	0-40 **(≈2.70)**[c]	0 **(38)**[c]	0-40 **(≈90)**[c]	0-40 **(0.80-0.86)**[c]	0-80 **(0.47-0.54)**[c]
Ethanol	40 **(≈3.11)**[c]	0 **(36)**[c]	0-150 **(≈90-100)**[c]	0-10 **(0.80)**[c]	0-10 **(0.47)**[c]
pH	6 **(3.10)**[b,d]	7 **(33.3)**[b,e]	6 **(201)**[b,d]; 8.2 **(33)**[c]	8 **(1.50)**[b,d]	7 **(1.13)**[b,e]
Gas-to-liquid ratio	12 **(3.36)**[c]	ND	12 **(99)**[c]	ND	ND
Intermittent headspace flushing with N_2	YES **(3.52)**[f]	YES **(42)**[f]	YES **(184)**[f]	YES **(1.68)**[f]	YES **(0.51)**[f]
Continuous sparging with N_2	YES **(3.10)**[g]	NSD	YES **(201)**[g]	NSD	NO **(1.13)**[h]

ND, not determined; NSD: no significant difference
a) YE and tryptone supplemented simultaneously to the medium
b) in pH-controlled condition
c) in non-pH-controlled condition
d) with continuous N_2 sparging
e) without continuous N_2 sparging
f) manual pH correction by 3M NaOH pulse
g) pH 6
h) pH 7

Substrate concentration is an important factor for H_2 production by anaerobic fermentation. Operating at high substrate load is one of the main requirements for an economically sustainable H_2 production process based on dark fermentation (Ljunggren and Zacchi 2010), but at the same time can negatively affect the bacterial metabolism resulting in a reduction of H_2 yield and/or productivity (Van Niel et al. 2003, Van Ginkel and Logan 2005b, O-Thong et al. 2008c, Frascari et al. 2013, Paper **III**). The initial substrate concentration had an effect on growth and H_2 production in *C. celer*. The highest cell growth (0.54 g_{CDW}/l), specific growth rate (0.53 h^{-1}), H_2 accumulation (101.2 mmol H_2/l) and H_2 yield (2.95 mol H_2/mol glucose) were achieved at 50 mM of glucose (Paper **III**). According to the Eq. 6, the maximum apparent growth and H_2 production rates were found at 35.3 and 54.5 mM of glucose, respectively. The inhibiting effect of glucose at high concentration is discussed in section 4.3.

In the experimental setup chosen for the optimization of fermentation parameters (i.e. batch cultures in closed serum bottles) the production of organic acids during glucose fermentation caused a significant drop of the culture pH completely inhibiting growth at pH< 5.5 (Paper **I**). Buffers are often used in small-scale laboratory studies to control the pH and minimize the effect of medium acidification. The use of phosphate buffer up to 50 mM alleviated the medium acidification drastically improving H_2 accumulation and glucose degradation by 2.5- and 4-fold, respectively (Paper **I**). However, growth was completely inhibited at phosphate buffer concentrations over 50 mM. Comparison between different buffering agents (phosphate buffer, carbonate buffer and HEPES) showed that under the tested conditions (i.e. at 50 mM buffer and 50 mM glucose, in a non-pH-controlled system) none of buffers was able to prevent excessive medium acidification that in turn resulted to be the main cause for growth inhibition and incomplete substrate degradation (Paper **II**). When the pH was manually adjusted by pulsing NaOH in the medium, all the buffers performed similarly in terms of H_2 production. Thus, pH control by addition of caustic agents was a more critical factor than the nature of the buffering system. Given the performance and the economical convenience for lab-scale investigations, phosphate buffer was used in all the experiments in this study.

Yeast extract and other proteic hydrolysates (e.g. tryptone and peptone) are often supplemented in growth media to aid bacterial growth and bioconversion activities by providing essential building blocks (e.g. peptides, free amino acids, trace elements and water soluble vitamins). Increasing the amount of YE and tryptone (up to 5 g/l each) boosted glucose degradation and growth rate in *C. celer*, but considerably decreased the H_2 yield that dropped by 2-fold from ≈3.0 to ≈1.5 mol H_2/mol glucose both at 25 mM and 50 mM of glucose (Paper **I**, our unpublished results). Based on these observations, the amount of YE and tryptone should be minimized to achieve high yield, whereas for high growth rate it should be increased to sustain substrate degradation and biomass synthesis. In this study, supplementing the medium with 2 g/l of each allowed to obtain high yields without compromising the fast growth of *C. celer*.

Hydrogenases are iron-sulfur proteins with two metal atoms at their active site, either two Fe atoms (in FeFe-H_2ases) or a Ni and an Fe atom (in NiFe-H_2ases) (Vignais and Billoud 2007). Therefore, for a correct assembly, maturation and function of these proteins an adequate amount of iron and nickel should be available in the growth medium. Supplementing the medium with an increasing amount of Fe^{2+} significantly improved H_2 production with the best accumulation and yield observed at 200 mg/l of $FeSO_4 \cdot 7H_2O$ (Table 19) (Paper **I**), corresponding to 144 μM of Fe^{2+}. This concentration is several folds higher than the estimated 25-40 μM required for fermentative growth (van Niel et al. 2011). In contrast, the concentration of NiCl in the medium did not

affect the H_2 production in *C. celer* (our unpublished results), suggesting that nickel amount in the original medium is sufficient for NiFe-H_2ases to be functional.

The presence of end-products in the growth medium can trigger a metabolic response at several levels including the central carbon metabolism (Nicolaou et al. 2010). Consequently, they can inhibit or even stimulate the production of the desired end-product also at subinhibitory concentrations (Grupe and Gottschalk 1992, He et al. 2009, Rydzak et al. 2011, Tang et al. 2012, Paper **III**). The addition of a wide range of acetate (20-150 mM) and ethanol (40-150 mM) to the medium improved the H_2 yield (Paper **III**). The highest yields observed were 3.0 mol H_2/mol glucose at 40 mM of acetate and 3.11 mol H_2/mol glucose at 40 mM of ethanol. Formate addition instead did not have any positive effect on H_2 yield. It can be hypothesized that under stressful conditions caused by the presence of high concentration of soluble metabolites the energy for cellular maintenance increases and thus the metabolic flux is directed through reactions associated with ATP generation such as acetate synthesis that can be linked to H_2 production. Unlike the yield, H_2 productivity and growth rate were severely inhibited by the addition of the three soluble metabolites (Paper **III**). Their inhibiting effect at high concentration is discussed in section 4.3.

The culture pH is an important variable of the anaerobic fermentation process that affects growth and substrate consumption rates as well as end-product synthesis profiles. The optimal pH for H_2 production in *C. celer* resulted to be dependent on the experimental setup (Paper **I**, **IV**). In closed batch cultures under non-pH-controlled conditions the optimal pH for H_2 accumulation was observed to be 8.2 at 67 °C (approximately 9.0 at room temperature) in accordance with the optimal pH previously reported for growth (Engle et al. 1996). However, in this experimental setup the culture pH quickly dropped to a critical value due to the production of organic acids during fermentation, resulting in the inhibition of both growth and H_2 production (Paper **I**-**III**). Therefore, only the optimal initial pH could be determined. In case of the alkalitolerant *C. celer*, setting a higher initial pH value delays the detrimental effects of medium acidification improving the substrate consumption and the accumulation of H_2. On the contrary, in pH-controlled conditions pH can modulate the performance of the H_2 production process by *C. celer*. At moderately acidic pH the conversion of glucose to H_2 was more favorable (>2 mol H_2/mol glucose) than at neutral/alkaline pH (<2 mol H_2/mol glucose), whereas growth rate and volumetric H_2 productivity increased as pH was shifted to more alkaline levels (Paper **IV**). As a consequence, acidic pH should be preferred for a high yield-oriented process, while alkaline pH for a high productivity-oriented process.

The accumulation of H_2 during fermentation both in liquid and gaseous phases is detrimental for the overall efficiency of the hydrogenogenic process due to the

inhibition of H_2ases and redirection of the metabolism towards the synthesis of other reduced end-products (Schröder et al. 1994, Soboh et al. 2004, Willquist et al. 2011, Bielen et al. 2013a). The effect of H_2 concentration in the fermentation vessel on H_2 production in *C. celer* was assessed by employing several experimental setups, i.e. different headspace-to-culture volume ratios (Paper **I**), intermittent headspace flushing with N_2 (Paper **II**) and continuous sparging with N_2 in CSTR (Paper **IV**). All experiments clearly indicated that the H_2 yield was severely affected by H_2 accumulation. Specifically, the H_2 yield remarkably improved when the H_2 concentration in the system was kept at low level (Table 19) (Paper **I**, **II**, **IV**). On the other hand, growth rate and H_2 productivity were either minimally (Paper **II**) or completely not affected (Paper **IV**). In addition, *C. celer* was able to tolerate high H_2 concentration (up to a P_{H2} of 223 kPa) without being completely inhibited either in growth or in H_2 production. The H_2 tolerance exhibited by *C. celer* towards H_2 build-up is attractive because H_2 production should be achieved preferably without the need for sparging gas to reduce capital costs for gas-upgrading process (Ljunggren and Zacchi 2010).

4.3. Inhibitory effects of substrate and soluble end-products

In this section, growth and H_2 production kinetics are described to assess the inhibitory effect of substrate load and soluble metabolites (i.e. acetate, formate and ethanol), whereas their concentrations for optimal growth and H_2 production have been described in section 4.2. Growth and H_2 production in *C. celer* were only minimally affected by initial glucose concentration up to 100 mM, whereas inhibition was observed with further increment of substrate, especially on biomass formation and specific growth rate (Paper **III**). The inhibition of H_2 production at high substrate concentrations has been previously reported for other thermophilic H_2-producing microorganisms (Van Niel et al. 2003, Frascari et al. 2013). Despite the dramatic reduction in growth rate and the consequent increase of the incubation time at the highest glucose concentration tested (300 mM), *C. celer* was still capable of maintaining a reasonable H_2 production yield (1.98 mol H_2/mol glucose). The critical glucose concentrations ($[S]_{crit}$) estimated for growth and H_2 production rates with a noncompetitive inhibition equation (Eq. 6) were 292 and 358 mM, respectively (Table 20). Despite possible differences in osmolality in the culture due to different medium composition, *C. celer* showed a similar sensitivity towards the substrate to the one found in *Ca. saccharolyticus* cultured on sucrose ($[S]_{crit}$= 292 mM) (Van Niel et al. 2003).

Table 20. Summary of estimated parameters (K_c, inhibition constant; C_{50}, half maximal inhibitory concentration; CIC, critical inhibitory concentration) used to assess the inhibition kinetics of specific growth rate and volumetric H_2 productivity as function of different inhibitors pertaining to C. celer´*s cultures. As a comparison, estimated parameters for the closely related species* Ca. saccharolyticus *are reported.*

Inhibitor	*C. celer*[a]						*Ca. saccharolyticus*[b]
	Specific growth rate			H_2 productivity			H_2 productivity
	K_c[c] (mM)	C_{50}[d] (mM)	CIC[e] (mM)	K_c[c] (mM)	C_{50}[d] (mM)	CIC[e] (mM)	CIC[e] (mM)
Sodium acetate	107	126	-	57	80	496[f];510[g]	192
Sodium formate	113	116	-	77	88	-	-
Ethanol	55	56	-	83	162	-	-
Potassium acetate	-	-	-	-	-	417	206
Sodium chloride	-	-	-	-	-	467	216
Potassium chloride	-	-	-	-	-	407	250
Sodium lactate	-	-	-	-	-	-	184
Substrate	-	-	292[h]	-	-	358[h]	292[i]
Total solutes	-	-	-	-	-	532–622[j]	400–425[j]

a) data obtained from Paper **III**
b) data obtained from van Niel et al. 2003
c) estimated with Eq. 7
d) estimated with Eq. 8
e) estimated with Eq. 6 and Eq. 9
f) at pH 8
g) at pH 6.5
h) estimated with glucose as substrate
i) estimated with sucrose as substrate
j) includes also monovalent ions and substrate concentrations

Although H_2 yield and accumulation resulted unaffected and in some case even improved in presence of a wide concentration range of soluble metabolites (up to 80-150 mM depending on the tested metabolites), growth and H_2 productivity were more sensitive towards the addition of exogenously added metabolites (Paper **III**). Modeling the inhibitory effect of acetate, formate and ethanol with two inhibition models (Eq. 7 and Eq. 8) revealed that in *C. celer* the three soluble metabolites did not have the same effect on growth and H_2 production rates. Ethanol had a higher inhibitory effect on the specific growth rate being the estimated K_c (inhibition constant) and C_{50} (half maximal inhibitory concentration) for ethanol 2-fold lower compared to those of acetate and formate (Table 20). Similarly, in *Cl. thermocellum* the C_{50} for ethanol was found to be about 2.5-fold lower than for acetate (Herrero et al. 1985a, Herrero et al. 1985b). On the other hand, when the H_2 production kinetics was taken into account, acetate and formate had a higher inhibitory effect compared to ethanol. Especially, the estimation of C_{50} for acetate and formate was two times lower than for ethanol (Table 20). The higher inhibitory power of acetate over ethanol towards H_2 production was observed also in *Et. harbinese* B49, where C_{50} was estimated to be 62 mM for acetate and 154 mM for ethanol (Tang et al. 2012).

Although at high concentrations all three soluble metabolites can affect cell growth and H_2 synthesis, under optimal conditions for H_2 production acetate accounted for up to 78 % of the total soluble metabolites in the fermentation broth (Paper **II**). Therefore, it is

more likely that the accumulation of this metabolite in the culture may represent a threat for *C. celer*. The similar critical inhibitory concentrations between acetate salts (i.e. Na-acetate and K-acetate) and their corresponding chloride salts (i.e. NaCl and KCl), and between Na-acetate at different pH (Table 20) suggests that the inhibition of H_2 productivity (and most probably of growth rate) by acetate accumulation occurs due to the increase of the ionic strength in the medium rather than due to the uncoupling effect of the undissociated form.

An ideal H_2-producing microorganism should tolerate high concentrations of substrate and end-products (Pawar and Van Niel 2013). However, the results of this study suggest that, like *Ca. saccharolyticus* (Van Niel et al. 2003), *C. celer* is not particularly osmotolerant being their respective critical substrate concentration and critical solute concentration in the same order of magnitude (Table 20).

4.4. A genomic insight into the carbon and energy metabolism

The whole genome of *C. celer* was sequenced and analyzed through a comparative genomics approach in order to gain insight into carbon and energy metabolism for a more systematic investigation of the potential of this organism for bioenergy applications (Paper **V**, **VI**). The "improved high-quality draft" genome (GenBank accession no. CAVN010000000) has a total size of 2,644,756 bp, organized in 56 scaffolds (>1 kb) (consisting of 162 contigs with an N_{50} of 128,968 bp), the longest being 1,976,539 bp. The G+C content of the genome is 31.3 %. The genome contains 2,535 annotated genes (locus tag in the text reported as 0XXXX instead of TCEL_0XXXX) with 2,381 predicted to be protein-coding sequences (CDS), 72 % of which have been assigned with putative biological functions and 28 % are hypothetical proteins. The genome is predicted to contain 153 RNA genes: 45 rRNA genes (15 5s, 15 16s and 15 23s) and 108 tRNA genes. Genome size, G+C content and number of CDS are consistent with those of other *Caloramator* species (Table 21).

Table 21. General features of C. celer *genome compared to members of* Caloramator *genus with a sequenced genome and other thermophilic H_2 producers (modified from Van De Werken et al. 2008).*

Organism	DNA scaffolds	Size (Mbp)	CDSs	GC (%)	rRNA operons	tRNA genes
C. celer DSM 8682	56	2.644	2381	31.3	15	109
C. boliviensis DSM 22065	20	3.200	2896	32.0	NR	41
C. australicus RC3	31	2.654	2767	32.8	3	62
C. sp. ALD01	65	2.530	2475	31.4	NR	57
Ca. saccharolyticus DSM 8903	1	2.970	2679	35.3	3	46
Cl. thermocellum ATCC 27405	1	3.843	3191	39.0	4	56
Cal. subterraneus subsp. *tengcongensis MB4*	1	2.689	2588	37.6	4	55
T. maritima MSB8	1	1.861	1858	46.3	1	46
P. furiosus DSM 3638	1	1.908	1983	40.8	1	46

NR: not reported

4.4.1. Carbohydrate transport and utilization

Analysis of the genome sequence showed that *C. celer* encodes both ABC-type transporters and PTS transporters for carbohydrate uptake as well as cationic symporters. According to the Transporter Classification Database (TCDB) designations, *C. celer* contains 26 putative genes belonging to the ABC Superfamily (TC:3.A.1) involved in carbohydrate transport, 10 putative genes encoding for elements of phosphotransferase system and only one for a cationic symporter. Based on the identification of all components of the EMP pathway and the lack in the genome of genes involved in the ED pathway or the oxidative branch of the PPP, *C. celer* most likely metabolizes hexoses to pyruvate through EMP pathway. The reductive branch of the PPP is however coded in the genome of *C. celer* (Figure 6), thus production of precursors for biomass synthesis from glucose through this metabolic route is possible. Comparison between the observed phenotype and the genome content confirms the previously reported capability of *C. celer* to consume glucose, sucrose, fructose, galactose and maltose, and the inability to convert pentose sugars, such as xylose and arabinose, as well as glucuronic acid to intermediates of the EMP pathway. Interestingly, the genome analysis reveals the potential for mannose and glycerol consumption, even though no growth has been observed on these substrates thus far. In *C. celer* the key enzymes of the EMP pathway that convert phosphorylated sugars to pyruvate are two PFKs (02267 ATP-dependent; 00490 uncertain specificity), a GAPDH (00702), a pyruvate kinase (00489) and possibly a pyruvate dikinase (01971) (Figure 6). The last step of the glycolysis, the conversion of PEP to pyruvate, can also occur indirectly via the “malate shunt”, a metabolic route that also serves as a source of reducing equivalents (NADPH) for biomass synthesis.

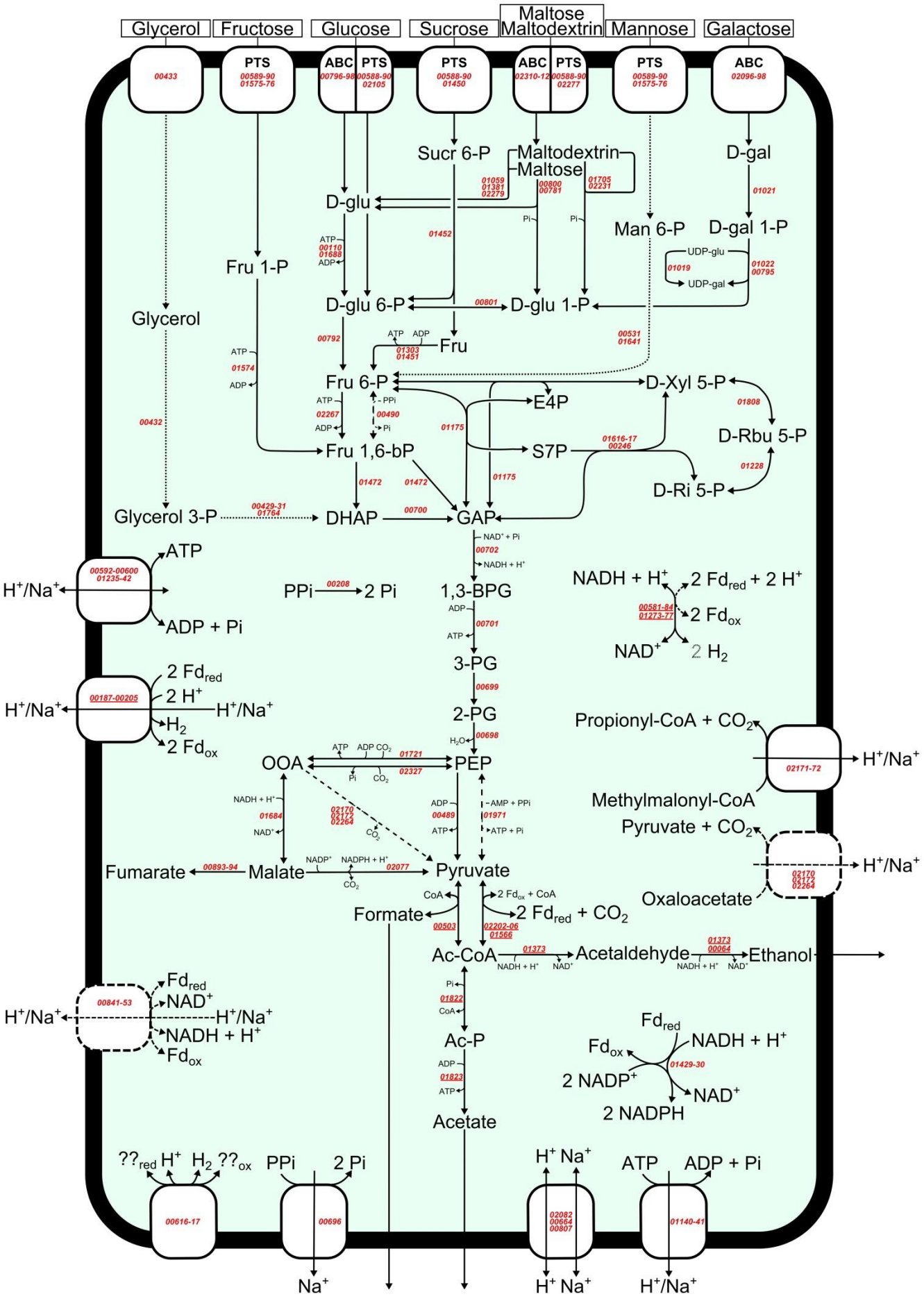
Glycerol
Fructose
Glucose
Sucrose
Maltose
Maltodextrin
Mannose
Galactose
PTS
ABC
Sucr 6-P
Maltodextrin
Maltose
D-gal
D-glu
Man 6-P
D-gal 1-P
UDP-glu
UDP-gal
Fru 1-P
D-glu 6-P
D-glu 1-P
Fru
Fru 6-P
D-Xyl 5-P
E4P
D-Rbu 5-P
S7P
Fru 1,6-bP
D-Ri 5-P
Glycerol
Glycerol 3-P
DHAP
GAP
ATP
ADP + Pi
PPi
2 Pi
1,3-BPG
3-PG
2-PG
NADH + H+
2 Fdred + 2 H+
2 Fdox
NAD+
2 H2
2 Fdred
2 H+
H2
2 Fdox
OOA
PEP
Propionyl-CoA + CO2
Methylmalonyl-CoA
Pyruvate + CO2
Oxaloacetate
Fumarate
Malate
Pyruvate
Formate
2 Fdred + CO2
Ac-CoA
Acetaldehyde
Ethanol
Fdred
NAD+
NADH + H+
Fdox
Ac-P
Acetate
2 NADP+
2 NADPH
??red H+ H2 ??ox
PPi
2 Pi
H+ Na+
ATP
ADP + Pi
Na+
H+/Na+

Figure 6. Reconstructed metabolic network of the central carbon metabolism of C. celer. *The locus tags (reported as 0XXXX and not as TCEL_0XXXX) of the putative genes associated with the reactions are given. Putative genes whose expression has been confirmed by qPCR (Paper **III**) are underlined. Dotted lines represent reactions for utilization of substrates that do not to sustain growth in* C. celer. *Dashed lines represent reactions for which the corresponding genes have been identified but whose function could not be inferred on the basis of sequence analyses and/or literature searches. For clarity, the putative complete pathway for butyrate synthesis is not reported in the figure, but the genes involved are reported in Paper **VI**. Abbreviations: 1,3-BPG, 1,3-bisphosphoglycerate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; Ac-CoA, acetyl coenzyme A; Ac-P, acetyl-phosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CoA, coenzyme A; D-gal 1-P, D-galactose 1-phosphate; D-gal, D-galactose; D-glu, D-glucose; D-glu 1-P, D-glucose 1-phosphate; D-glu 6-P, D-glucose 6-phosphate; DHAP, dihydroxyacetone phosphate; D-Rbu 5-P, D-ribulose 5-phosphate; D-Ri 5-P, D-ribose-5-phosphate; D-Xyl 5-P, D-xylulose-5-phosphate; E4P, erythrose 4-phosphate; Fd_{ox}, ferredoxin (oxidized form); Fd_{red}, ferredoxin (reduced form); Fru 1,6-bP, fructose 1,6-biphosphate; Fru 1-P, fructose 1-phosphate; Fru 6-P, fructose 6-phosphate; Fru, fructose; GAP, glyceraldehyde 3-phosphate; Glycerol 3-P, glycerol 3-phosphate; Man 6-P, mannose 6-phosphate; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); OOA, oxaloacetate; PEP, phosphoenolpyruvate; Pi, orthophosphate; PPi, pyrophosphate; S7P, sedoheptulose-7-phosphate; Sucr 6-P, sucrose 6-phosphate; UDP-gal, galactose-uridine diphosphate; UDP-glu, glucose-uridine diphosphate.*

4.4.2. Pyruvate catabolism

Fermentation of carbohydrates leads to the formation of pyruvate, a key intermediate in the central carbon metabolism of *C. celer*. Given the lack in the genome of putative genes encoding for lactate dehydrogenase and alanine aminotransferase for the respective synthesis of lactate and alanine, all the pyruvate must be converted to acetyl-CoA. In *C. celer* the conversion of pyruvate to acetyl-CoA can occur either via the oxidative decarboxylation of pyruvate to acetyl-CoA, CO_2 and Fd_{red} catalyzed by two putative PFORs (02202-02206; 01566), or via the non-oxidative generation of acetyl-CoA and formate from pyruvate catalyzed by a putative PFL (00503) (Figure 6). Acetyl-CoA produced from pyruvate catabolism can be converted to acetate by a two-step reaction catalyzed by a putative PTA (01822) and a putative ACK (01823) yielding one additional ATP to the two already produced during the glycolysis (Figure 6). *C. celer* encodes also a complete pathway for butyrate synthesis which potentially involves the consumption of reducing equivalents (NADH) as well as production of ATP. However, during glucose fermentation butyrate production is minimal (<1 mM) or in most cases not detectable (Paper **I**-**IV**). Alternatively, acetyl-CoA can be the substrate for the two-step reduction to ethanol mediated by a putative bifunctional ALDH/ADH (01373) and a putative ADH (00064) (Figure 6). This reaction consumes two NAD(P)H and thus serves as a possible route for *C. celer* to dispose of reducing equivalents.

4.4.3. Hydrogenases and electron shuttling systems

Reducing equivalents produced during glycolysis and oxidative decarboxylation of pyruvate (NADH and Fd_{red}, respectively) can be recycled through H_2 production catalyzed by H_2ases. Four H_2ases were identified in the genome of *C. celer*: two FeFe-H_2ases and two NiFe-H_2ases (Figure 6, 7A, 7B, 7C). The putative FeFe-H_2ases (00580-00584; 01273-01277) are cytosolic heterotetrameric complexes whose subunits have high homology to those of the FeFe-H_2ase characterized in *Cal. subterraneus* subsp. *tengcongensis* (TTE_0890-0894) (Soboh et al. 2004). In addition, the gene arrangement of the two FeFe-H_2ase clusters in *C. celer* is similar to the one described for TTE_0890-0894 (Figure 7A). Recently, this five-gene operon has been proposed to encode a potential bifurcating FeFe-H_2ase, based on the high homology of three subunits with the trimeric bifurcating FeFe-H_2ase of *T. maritima* (Schut and Adams 2009). The latter H_2ase couples the thermodynamically unfavorable oxidation of NADH to H_2 production through utilization of the exergonic oxidation of Fd_{red}. However, biochemical characterization of the H_2ase encoded by TTE_0890-0894 showed that this enzyme possesses only NADH-dependent hydrogenase activity (Soboh et al. 2004). According to these observations, it is likely that the two clusters in *C. celer* encode putative NADH-dependent H_2ases (Figure 6), although the bifurcating hydrogenase activity can not be ruled out.

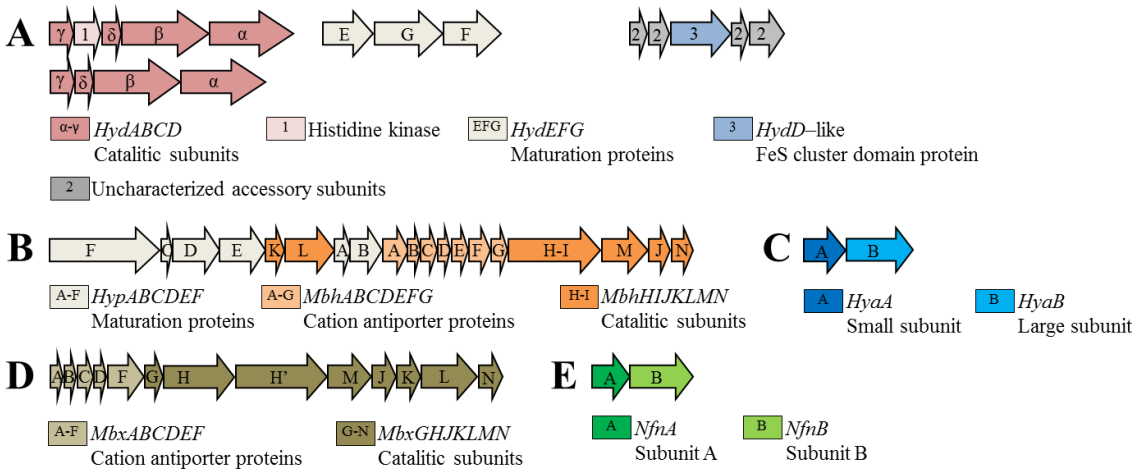

Figure 7. Clusters encoding two putative FeFe-H_2ases (00580-00584; 01273-01277), their corresponding maturation (01279-01281) and accessory genes (01285-01289) (A), a putative energy conserving NiFe-H_2ase complex (00187-00205) (B), a putative dimeric NiFe-H_2ase (00616-00617) (C), a putative energy-conserving ferredoxin:NAD(P)$^+$ oxidoreductase (00853-00841) (D) and a putative electron-bifurcating transhydrogenase (01429-01430) (E).

One NiFe-H_2ase is encoded by a 19-gene cluster (00187-00205) including 13 genes predicted to encode a membrane-bound multimeric complex and 6 genes putatively involved in the assembling of the NiFe center and the maturation (Figure 7B). The 13 subunits in the complex show from 30 to 54 % identity to genes PF_1423-1436 of *P.*

furiosus. In *P. furiosus* this cluster encodes a Fd-dependent membrane-bound NiFe-H_2ase (MBH) that conserves energy by generating an ion gradient across the membrane that can be used to generate ATP via a membrane-bound ATP synthase (Sapra et al. 2003). Based on sequence homology, it is likely that *C. celer* reoxidizes Fd_{red} by a similar energy-conserving mechanism (Figure 6). A second putative membrane bound NiFe-H_2ase (00616-00617) belonging to group I (Calusinska et al. 2010) was identified in the genome (Figure 6 and 7C), but role, direction of the reaction and redox partner(s) are difficult to infer due to the scarce information about this group of H_2ases in closely related organisms. No FHL complex was identified in the genome, thus *C. celer* can not evolve H_2 along with CO_2 from the dissimilation of formate.

Based on genome analysis, other electron shuttling systems can be potentially involved in the central carbon metabolism of *C. celer*. A *nfnAB* homolog (01429-01430) was identified in the genome of *C. celer* (Figure 6, 7E). The Nfn complex is an electron-bifurcating transhydrogenase that couples exergonic reduction of $NADP^+$ with ferredoxin to drive the endergonic reduction of $NADP^+$ with NADH (Wang et al. 2010, Huang et al. 2012). Together with the malate shunt, this enzyme would provide NADPH for biomass synthesis (Figure 6). A 13-gene cluster (00853-00841) with good amino acid identity and similar gene organization to the *mbx* cluster of *P. furiosus* (PF_1441-1453) (Schut et al. 2007) is the only possible candidate to code for an enzyme with a putative energy-conserving ferredoxin:$NAD(P)^+$ oxidoreductase activity (Figure 6 and 7D). However, in *P. furiosus* the MBX complex was proposed to take part only in sulfur metabolism and thus its role in *C. celer* remains unclear.

4.4.4. Energy metabolism

Transmembrane ion gradients, both in form of proton and cation, are crucial for driving endergonic reactions such as solute transport and ATP synthesis. The genome of *C. celer* encodes several putative Na^+ pumps (i.e. methylmalonyl-CoA decarboxylase, energy-consuming Na^+ pump and Na^+-exporting V-type pyrophosphatase), putative H^+-translocating enzymes (MBH and MBX complexes) and putative cation exchange antiporters (NhaC and NapA family) (Figure 6). Therefore, *C. celer* should be able to establish both H^+ and Na^+ ion-motive forces and use cation exchange antiporters to balance the two gradients.

In *C. celer* ATP is mainly produced by substrate-level phosphorylation at three stages: in the glycolysis during the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate and of phosphoenolpyruvate to pyruvate, in the first step of the malate shunt via an ATP-linked PEP carboxykinase, and in the fermentation pathway during the synthesis of acetate. As discussed above, *C. celer* can potentially establish ion gradients through a set of ion-translocating enzymes and exploit them for ATP synthesis

via a putative A/V-type ATP synthase (00592-00600) or a putative F_0F_1-ATP synthase 01235-01242) (Figure 6). In contrast to what reported for other phylogenetically related organisms (Heinonen and Drake 1988, Bielen et al. 2010), several genomic as well as physiological observations, i.e. the lack of putative PPi-dependent PFK, the presence of a putative cytosolic pyrophosphatase (00208), the low intracellular PPi (our unpublished results) and the high anabolic rates, suggest that PPi might not be a primary energy currency.

4.5. Fermentative and energy metabolism

Fermentation conditions can dramatically affect the carbon and electron flow and in turn the end-product yields during anaerobic fermentation. Factors pertaining to the fermentative process known to alter the metabolic fluxes are media composition, substrate composition and load, H_2 and CO_2 concentration, pH, growth phase, osmolality and accumulation of soluble metabolites. For a certain organism the genome content (Carere et al. 2012) and the regulatory mechanisms that modulate gene expression and enzymatic activities (Soboh et al. 2004, Stevenson and Weimer 2005, Willquist and van Niel 2010, Bielen et al. 2013b) dictate the changes of the internal fluxes and how they translate into the end-product profile. The modifications of metabolic fluxes mainly aim at the maintenance of cellular redox and/or energetic homeostasis. In this study a comprehensive investigation of fermentative and energy metabolism of the thermophilic alkalitolerant bacterium *C. celer* was carried out by combining fermentation data, genomic data, transcription data, reconstruction of central carbon metabolism and metabolic flux analysis (MFA). This section summarizes the main findings related to changes in carbon flux and electron distribution, possible regulating mechanisms of the catabolic fluxes and energy conservation strategy in *C. celer*.

4.5.1. Assessment of metabolic flux distribution as affected by fermentation conditions

In this study the fermentative metabolism of *C. celer* was found to shift in response to several modifications of the culture conditions namely growth rate, growth phase, iron content in the medium, substrate availability, presence of soluble metabolites, pH and H_2 concentration. A recurring trend of great relevance for the interpretation of the results was observed throughout the study. Specifically, formate synthesis was directly correlated to the growth rate during glucose fermentation (Paper **I**-**IV**). Additionally, high formate flux was essential to sustain high growth rates (our unpublished results). Indeed, the formate yield and/or flux were always found to be high when the fermentation conditions favored elevated growth rates. This scarcely documented

phenomenon has several important implications for the efficiency of the H_2 production process in *C. celer*: i) rerouting the carbon and electron flow through PFL reduces the H_2 yield since this reaction does not supply Fd_{red} and formate is not further converted to H_2 and CO_2; ii) high growth rates and efficient H_2 production are not compatible. Given the aforementioned correlation, it is not surprising that formate synthesis occurred predominantly in the exponential phase of growth (Paper **I**-**IV**). Although ethanol synthesis was not directly correlated to the growth rate, its accumulation always started in exponential phase and under conditions favoring H_2 production it ceased at the beginning of the stationary phase. A growth phase-dependent breakdown of the fermentations under optimal conditions showed that in the exponential phase H_2 yield was limited due to the simultaneous production of formate and ethanol, whereas in the stationary phase ethanol, formate and biomass synthesis were minimal or absent, and all the glucose metabolized at this stage was stoichiometrically converted to acetate and H_2 (Figure 8H) (Paper **II**, **IV**). This observation is in contrast with the behavior described for *Ca. saccharolyticus* in which the conversion of glucose to H_2 was associated with the exponential phase of growth (Willquist et al. 2010).

The iron content in the growth medium had an impact on the end-product profile. Iron-limited conditions induced a reduction in H_2 and acetate yield by 19 and 23 % respectively and an increase of ethanol yield by 63 %, whereas formate yield was not affected (Figure 8A) (Paper **I**). These results suggest that under iron limitation, a condition reported to limit the synthesis and the activity of H_2ases (Junelles et al. 1988), *C. celer* partially redirected carbon and electron flux towards ethanol synthesis, employing this route as an alternative route for reductant disposal.

Supplying the medium with high YE and tryptone concentrations had a detrimental effect on H_2 and acetate yields that decreased by 50 and 30-40 % respectively, whereas both ethanol and formate yield increased by 2- to more than 3-fold (Figure 8B). The decline of H_2 yield in richer media was most likely caused by the increased growth rate and glycolytic flux that triggered a redirection of carbon and electron flux towards synthesis of ethanol and formate that in *C. celer* competes with H_2 production. Similarly, the decline of H_2 and acetate yield in favor of other reduced end-products at high growth rate and glycolytic flux was observed in other organisms during fermentation of simple sugars (Strobel 1995, Guedon et al. 1999, Stevenson and Weimer 2005, De Vrije et al. 2007, O-Thong et al. 2008c, Willquist et al. 2011).

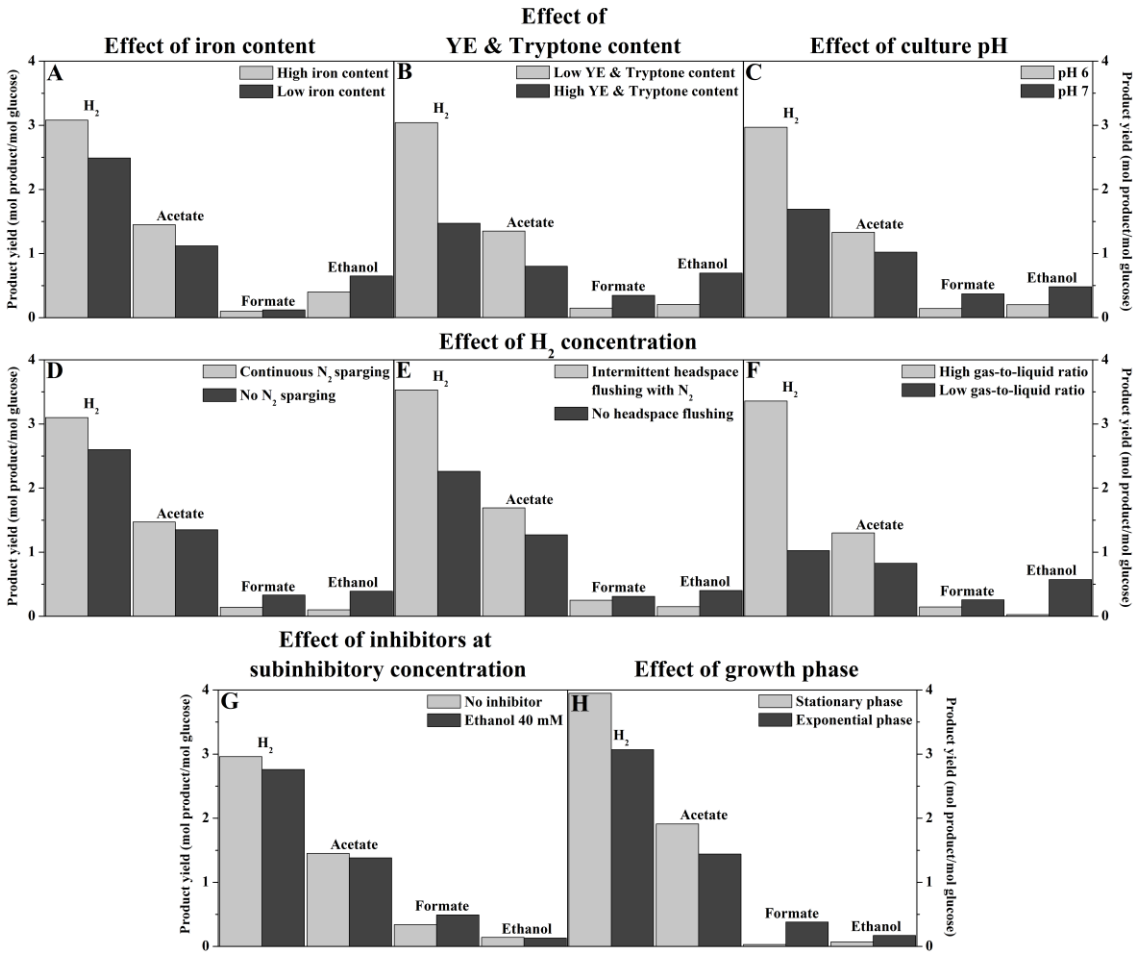

*Figure 8. Summary of changes in end-product profile in response to fermentation conditions. Each figure represents the end-product yields in the best and worst condition for H_2 production efficiency. A, B: performed in non-controlled batch bottles (Paper **I**); C-D: performed in controlled CSTR (Paper **IV**); E: performed in non-controlled batch bottles (Paper **II**); F: performed in non-controlled batch bottles (Paper **I**); G: performed in non-controlled batch bottles (Paper **III**); H: performed in non-controlled batch bottles (Paper **II**).*

Subinhibitory concentrations of exogenous end-products redirected the carbon and electron flow at the pyruvate and acetyl-CoA nodes resulting in changes in the metabolite profile (Figure 8G) (Paper **III**). The formate yield followed a growth-dependent trend being higher in the control fermentation (i.e. without addition of soluble metabolites), while ethanol yield increased by 2-fold when organic acids were added. The increase of ethanol production upon acetate addition has been reported by several other studies (Jones and Woods 1986, He et al. 2009, Rydzak et al. 2011, Shen et al. 2013). As recently shown by Shen et al. (2013), in *Ca. saccharolyticus* extracellular acetate can be converted by the cell to ethanol possibly as a means of detoxification. Despite the significant changes in the synthesis of metabolites competing with H_2 production, H_2 yield displayed only minimal variations (i.e. + 4 %, + 7 % and - 7 % in presence of subinhibitory concentration of exogenous acetate, ethanol and

formate, respectively), possibly due to overflow of pyruvate observed in this specific experiment that limited the available carbon and electrons for acetate and H_2 synthesis.

An analysis of the intracellular fluxes and end-product distribution clearly indicated that *C. celer* exhibited different metabolic patterns depending on the culture pH (Paper **IV**). Surprisingly, at moderately acidic pH the conversion of glucose to H_2 was more favorable (>2 mol H_2/mol glucose) than at alkaline to neutral pH (<2 mol H_2/mol glucose) as a consequence of the lower synthesis of other reduced by-products (i.e. formate and ethanol) with a notable transition in the metabolic behavior observed between pH 7 and 6 (Figure 8C). In particular, both formate yield and formate flux during exponential phase were more than 2-fold higher at neutral pH when also an elevated growth rate was observed. A widespread tendency to accumulate formate during glucose fermentation as culture pH becomes more neutral/alkaline was observed, albeit with different magnitude, among PFL-encoding organisms both in pure (Combet-Blanc et al. 1995, Kessler and Knappe 1996, Asanuma et al. 1999, Sridhar and Eiteman 2001, Even et al. 2003, Koussémon et al. 2003, Liu et al. 2011, Su et al. 2011) and mixed cultures (Temudo et al. 2007, Lee and Rittmann 2009). However, in this case it remains unclear whether the shift of the catabolic flux towards PFL is strictly a pH-dependent event or a consequence of the high growth rate and glycolytic flux favored by neutral/alkaline pH. Chemostat experiments performed at alkaline pH but at low dilution rates would unravel the causes of this metabolic shift. Unlike formate, ethanol production did not display a pattern dependent on culture pH.

Experiments in both controlled (Paper **IV**) and uncontrolled conditions (Paper **I**, **II**) confirmed that the fermentative metabolism of *C. celer* is affected by the H_2 build-up in the system. At high $[H_2]_{aq}$ the decrease of H_2 and acetate synthesis was accompanied by the increased ethanol and formate production (Figure 8D, 8E and 8F), albeit these changes occurred to different extents depending on the experimental set-up. Therefore, in order to achieve efficient H_2 production $[H_2]_{aq}$ must be kept at low level. Metabolic shifts triggered by high $[H_2]_{aq}$ are a consequence of the inability of H_2ases to regenerate oxidized cofactors to sustain the catabolic flux. Consequently, alternative pathways are employed to dispose of excess reducing power. The next section will provide a detailed description on how *C. celer* manages reducing equivalents to achieve redox homeostasis and to sustain the catabolic metabolism.

4.5.2. Generation and consumption of reducing equivalents in response to H_2 concentration

The reconstructed central carbon metabolism of *C. celer* (Figure 6 and 9) indicates that while glycolysis via EMP pathway yields 2 NADH per one glucose metabolized, generation of Fd_{red} is dependent on flux distribution at the pyruvate node. At this node

pyruvate is non-oxidatively dissimilated to acetyl-CoA and formate by PFL or oxidatively decarboxylated to acetyl-CoA by PFOR. Only the latter generates Fd_{red} and when 100 % of the flux goes through PFOR, generation of reducing equivalents is maximized resulting in the formation of 2 NADH and 4 Fd_{red}. These reduced cofactors need to be reoxidized allowing the glycolytic flux to proceed.

In *C. celer* the oxidation of NADH can occur either by proton reduction via NADH-dependent FeFe-H_2ases (00580-00584; 01273-01277) or by ethanol synthesis via acetaldehyde/alcohol dehydrogenase (01373; 00064), while the oxidation of Fd_{red} relies only on the activity of the Fd-dependent MBH complex (00187-00205) (Paper **IV, VI**) (Figure 6 and 9). If all the reduced electron carriers were recycled by H_2ases, the complete oxidation of one molecule of glucose would yield 4 H_2 molecules. However, at a given temperature the oxidation of reduced cofactors by H_2ases is a function of the H_2 concentration (Verhaart et al. 2010, Bielen et al. 2013a). In particular, at high $[H_2]_{aq}$ the reoxidation of NADH via proton reduction becomes much more thermodynamically unfavorable than reoxidation of Fd_{red}, thus causing high accumulation of intracellular NADH. Since the activity of GAPDH, a key glycolytic enzyme, is strongly inhibited by high $NADH/NAD^+$ ratios (Lovitt et al. 1988, Payot et al. 1998, Payot et al. 1999, Willquist et al. 2011), this condition negatively affects growth rate and substrate consumption due to reduced glycolytic flux. In contrast, in *C. celer* growth rate and glycolytic flux were not affected by high $[H_2]_{aq}$ because intracellular NADH and $NADH/NAD^+$ were kept at low level. The complete NADH reoxidation, even under inhibiting conditions for NADH-dependent H_2 formation, was achieved by redirecting the carbon and electron flux to ethanol synthesis already during the exponential phase preventing the $NADH/NAD^+$ to increase and inhibit GAPDH (Figure 9) (Paper **IV**).

Although reoxidation of Fd_{red} via proton reduction is less sensitive to increased $[H_2]_{aq}$ (Bielen et al. 2013a), the higher formate yields observed in this study at elevated $[H_2]_{aq}$ suggest that excessive accumulation of Fd_{red} occurred due to inhibition of Fd-dependent H_2 synthesis, creating a bottleneck at PFOR (Figure 9) (Paper **IV**). Since no alternative reaction to H_2 synthesis is available for Fd_{red} oxidation and thermodynamics of the reaction catalyzed by PFL are independent of H_2 concentration, the redirection of the flux at the branched pyruvate node towards PFL was used by *C. celer* as a safety valve to relieve the cell from the burden of ferredoxin reoxidation in unfavorable conditions and thus avoiding a decrease in the metabolic flux at this catabolic step.

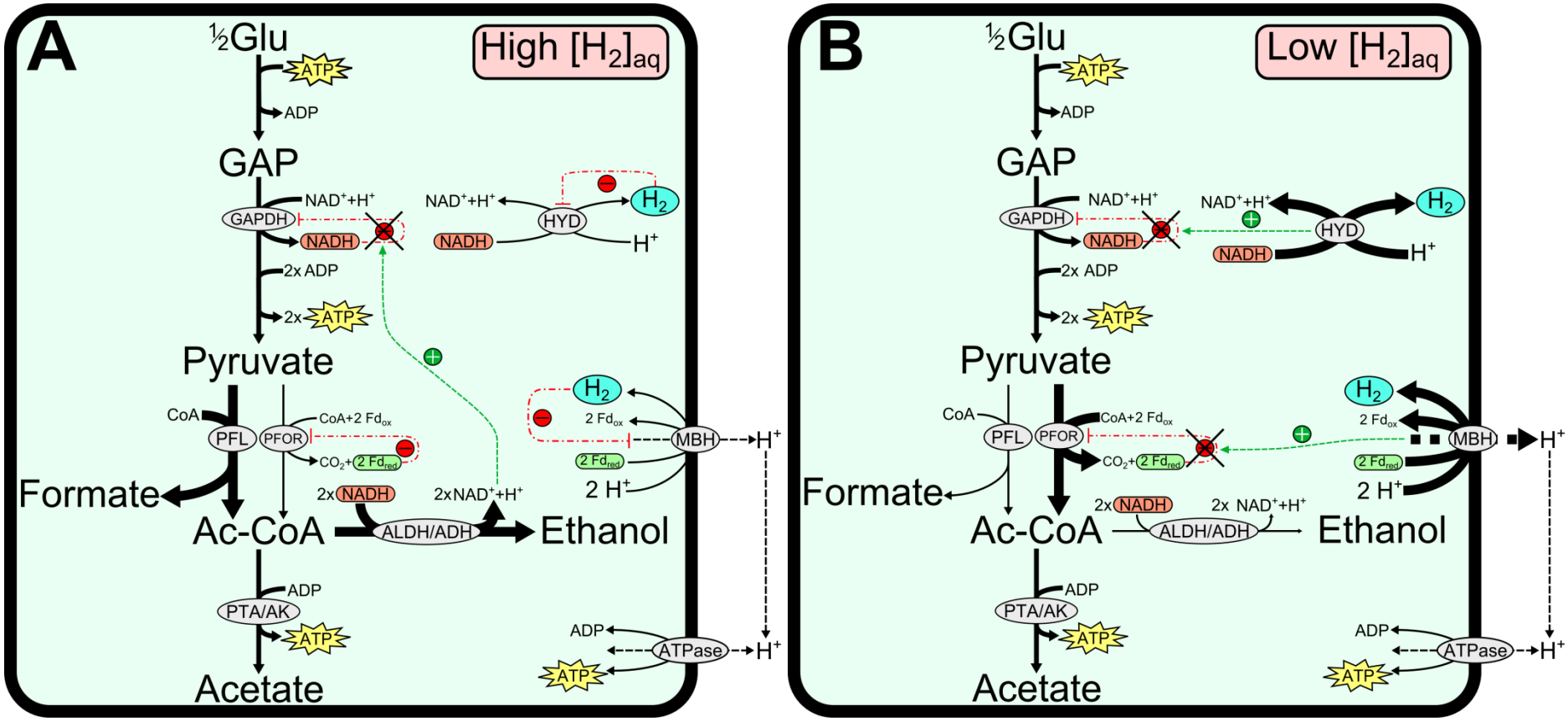

*Figure 9. Overview of the changes in the distribution of carbon and electron flux at high $[H_2]_{aq}$ (A) and low $[H_2]_{aq}$ (B). The size of the arrows depicts the variations of the fluxes through key catabolic enzymes measured in exponential phase during fermentations at pH 6 and pH 7 (Paper **IV**) (small-sized arrow, at least -15 % at both pH; medium-sized arrow, within ±15 % at both pH, big-sized arrow, at least +15 % at both pH). High $[H_2]_{aq}$ inhibits NADH- and Fd-dependent H_2ases, theoretically leading to accumulation of reduced cofactors. Partial redirection of carbon and electron flux towards the synthesis of ethanol and formate helps* C. celer *to bypass potential bottlenecks arising from inhibition of key reactions of the catabolic metabolism by high levels of reduced cofactors (A). At low $[H_2]_{aq,}$ most of reduced cofactors are reoxidized via proton reduction by NADH- and Fd-dependent H_2ases and consequently a reduction of ethanol and formate synthesis is observed (B).*

The results of this study proved that in *C. celer* both ethanol and formate synthesis provide an alternative to H_2 production for maintaining the redox balance and sustaining the catabolic flux when H_2ases are inhibited: ADH assists NADH-dependent H_2ase to keep low NADH levels, while PFL simultaneously supplies acetyl-CoA and stores reducing equivalents in formate thus regulating the pool of Fd_{red} available for Fd-dependent H_2ase (Figure 9). However, the production of formate and ethanol competes with H_2-evolving reactions for the disposal of reducing equivalents resulting in reduced H_2 yields (Paper **I**, **II**, **IV**). The complete reoxidation of both Fd_{red} and NADH through H_2 production was only observed under very specific conditions, i.e. when glucose was used for non-growth associated cell maintenance at low pH and $[H_2]_{aq}$ (Paper **II**, **IV**).

While the aforementioned metabolic scenario deduced by combining fermentation data, genomic data and MFA (Paper **IV**, **VI**) explains the experimental observations, it does not take into account the potential bifurcative nature of the two FeFe-H_2ases (00580-00584; 01273-01277), and the role of the putative energy conserving ferredoxin:$NAD(P)^+$ oxidoreductase (00853-00841) and the putative NADH-dependent ferredoxin:$NADP^+$ oxidoreductase (01429-01430) whose activities can not be accurately inferred simply on the basis of genomic analysis. Therefore, a more detailed knowledge of the electron shuttling systems is required to accurately predict the cellular electron-flow pathways in *C. celer*.

4.5.3. Regulation of the fluxes at key metabolic nodes

Mechanisms such as regulation of gene expression, allosteric regulation, post-translational modification, half-life of transcripts and corresponding enzymes, and thermodynamic constraints can govern the distribution of carbon and electron flux at metabolic branch points. Genome analysis revealed the presence of Rex binding motifs in the operator region of several genes involved in the central carbon metabolism (Paper **VI**). With the exception of acetate-synthesizing enzymes, the levels of all the enzymes putatively involved in end-product synthesis (i.e. two alcohol dehydrogenases, two hydrogenases, a pyruvate formate lyase and a pyruvate:ferredoxin oxidoreductase) might be transcriptionally controlled by the Rex regulator and consequently be dependent on the intracellular redox state. Further studies are needed to elucidate the members of the Rex regulon in *C. celer* and how they respond to changes of the intracellular redox state.

In this study the regulation of the key genes involved in pyruvate catabolism and product formation was investigated in response to the addition of subinhibitory concentrations of exogenous soluble metabolites (Paper **III**). Although the presence of end-products in the medium during *C. celer*'s growth triggered changes in the metabolite yields, minimal significant changes in gene regulation were observed (6

genes significantly regulated in presence of acetate and ethanol, and 1 in presence of formate) and not always in agreement with the measured yields. Only the expression of PFL and its activating enzyme showed a constant strong correlation with the formate yield as well as the growth rates, again providing proofs of the link between anabolic demand for growth and formate synthesis. Similarly, in *Cl. thermocellum* only minimal or no correlation between changes in product yields, and transcript and/or enzyme levels was found in response to addition of subinhibitory concentrations of end-metabolites and to different H_2 concentrations (Rydzak et al. 2011, Carere et al. 2014). Proteomic and transcriptomic studies in *Thermoanaerobacter* spp. also reported similar results, albeit observed in absence of inhibitors (Lin et al. 2011; Verbeke et al. 2014).

The lack of correlation between abundance of transcripts/proteins and end-product profile in *C. celer* and other fermentative thermophiles suggests that other factors besides gene expression are more prominently involved in the regulation of metabolic fluxes. For example, from the analysis of the fluxes at different culture pH it can be hypothesized that in *C. celer* the changes of carbon and electron flux at the pyruvate node might be function of: i) energy and anabolic demand, ii) allosteric regulation of the two enzymes at the branch point by substrate and product of the reaction, iii) optimal pH for enzymatic activity, iv) pH-dependent thermodynamics (Paper **IV**).

4.5.4. Energy conservation

Fermentative organisms employ several strategies for conserving energy derived from oxidation of the substrate: i) substrate-level phosphorylation (Gottschalk 1986), ii) electron transport phosphorylation (Gottschalk 1986), iii) utilization of inorganic PPi as energy carrier (Bielen et al. 2010), iv) electron bifurcation (Schut and Adams 2009). In this study, *C. celer* was cultured at high growth rates or under stress (i.e. at low pH or in presence of inhibitors) both conditions with high-energy requirements. Therefore, *C. celer* is expected to maximize energy recovery. Since synthesis of acetate allows the production of extra ATP via substrate-level phosphorylation (1 mol ATP/mol acetate) on top of the 2 mol ATP/mol glucose in the EMP pathway, the preference shown by *C. celer* throughout the study to direct the carbon and electron flux at the acetyl-CoA node to acetate production suggests that *C. celer* aims for maximization of ATP production.

Table 22. Stoichiometry, ATP gain and $[H_2]_{aq}$-dependent thermodynamics of the putative reactions of glucose fermentation in C. celer.

Eq.	Putative pathways in *C. celer*	ATP gain[a]	$\Delta G'_{70°C}$ at 1 M of H_{2aq}[b] (kJ/mol)	$\Delta G'_{70°C}$ at 10 µM of H_{2aq}[b] (kJ/mol)
14	$Glu+4H_2O \rightarrow 2Ace^- + 2HCO_3^- + 4H^+ + 4H_2$	4	-153.1	-284.5
15	$Glu+3H_2O \rightarrow 1Ace^- + 1Eth + 2HCO_3^- + 3H^+ + 2H_2$	3	-190.4	-256.1
16	$Glu+2H_2O \rightarrow 2Ace^- + 2For^- + 4H^+ + 2H_2$	4	-196.4	-262.1
17	$Glu+1H_2O \rightarrow 1Ace^- + 1Eth + 2For^- + 3H^+$	3	-233.8	-233.8

a) under the assumption that acetate yields 2 ATP, ethanol yields 1 ATP and PEP-dependent phosphotransferase system (PTS) is used for glucose uptake

b) calculations were modified from Bielen et al. 2013a

According to the reconstructed central carbon metabolism, four putative pathways for glucose catabolism are possible in *C. celer* (Table 22) (Paper **IV**, **VI**). Eqs. 14-15 direct 100 % of the carbon flux through PFOR, whereas Eqs. 16-17 direct the flux entirely through PFL. Conversion of glucose to acetate by Eq. 14 allows for maximal ATP generation (4 ATP) by completely relying on H_2ase activity to regenerate reduced cofactors (NADH and Fd_{red}). It has been hypothesized that the stoichiometric conversion of glucose to acetate, CO_2 and H_2 displayed by efficient thermophilic H_2 producers (e.g. *Ca. saccharolyticus* and *T. maritima*) serves as energy-conserving strategy to maximize the net ATP gain for fuelling energy demanding reactions (Willquist et al. 2010). However, at high H_2 concentration H_2ases are inhibited and less energy-efficient pathways for disposal of reducing equivalents need to be activated to sustain the catabolic flux. Indeed, at 70 °C and 1 M of dissolved H_2 this reaction is the least thermodynamically favorable ($\Delta G'_{70°C}$= -153.1 kJ/mol), but becomes more favorable as $[H_2]_{aq}$ decreases (Table 22). While reactions thermodynamically independent of the $[H_2]_{aq}$ used by other fermentative bacteria for balancing high $NADH/NAD^+$ (e.g. ethanol and lactate synthesis) yield only 2 ATP (Payot et al. 1999, Soboh et al. 2004, Willquist and van Niel 2010), the metabolic setup of *C. celer* allows for a reaction yielding 3 ATP (Eq. 17) whose $\Delta G'_{70°C}$ is completely independent of the $[H_2]_{aq}$. Thus, Eq. 17 ensures *C. celer* a higher energy-recovery from glucose catabolism and unaltered glycolytic flux even under unfavorable conditions for H_2 production. In addition, *C. celer* can potentially still obtain 4 ATP through Eq. 16 which is slightly more favorable at high $[H_2]_{aq}$ than Eq. 14.

Altering energy metabolism has been proposed as strategy to increase the H_2 yield (Nogales et al. 2012, Bielen et al. 2013a). This strategy would rely on lowering energy recovery from the glycolysis so that an increased metabolic flux to acetate would be required to generate the ATP needed to meet cellular energy requirements. Interestingly, H_2 production efficiency increased when *C. celer* was cultured at suboptimal extracellular pH, a condition expected to increase the energy demand. Indeed, the low intracellular ATP and the high ATP hydrolysis flux observed in this condition suggest that a substantial portion of the intracellular ATP was consumed possibly to achieve cytoplasmic pH homeostasis via H^+-ATPase (Paper **IV**). In this case non-growth-associated energy requirements increased forcing *C. celer* to maximize the net ATP via acetate synthesis. The fact that *C. celer* directed the flux through PFOR instead of PFL and that the estimated Fd-dependent H_2 flux increased (about +150 % in exponential phase) suggests that the putative MBH homolog (00187-00205) might play a role in the ATP generation via electron transport phosphorylation as expected by its inferred function (Paper **V**, **VI**). Indeed, in *P. furiosus* the formation of H_2 by MBH is able to add 1.2 ATP to the energy pool for each oxidized glucose molecule (Sapra et al. 2003). If the putative Fd-dependent H_2ase in *C. celer* had the same function as in *P. furiosus*, the increase of Fd-dependent H_2 flux would represent a strategy to boost energy

harvesting from glucose under high cell maintenance requirements. Based on this reasoning, increasing cellular energy demand or vice versa lowering the energy conservation efficiency might be a promising path to enhance H_2 yield by metabolic engineering.

This study also reveals several clues suggesting that in *C. celer* H_2 synthesis is not coupled with high anabolic activity, namely i) the high formate production at high growth rate preventing generation of Fd_{red} for H_2 production, ii) the possible non-involvement of PPi in catabolic metabolism, iii) the electron flow mainly directed to H_2 at reduced growth rates or in stationary phase. In *Ca. saccharolyticus* instead a strong link exists between anabolism and catabolism, mainly due to the important role that both ATP and PPi play as energy carriers and as regulators of key enzymes in the metabolic network (Bielen et al. 2010, Willquist and van Niel 2010, Willquist et al. 2011).

5. CONCLUSIONS

This study demonstrates the potential of the novel alkalithermophile *Caloramator celer* for fermentative biohydrogen production. A better understanding of the fermentative and energy metabolism was obtained using glucose as a model substrate by altering the metabolic fluxes through manipulation of fermentation conditions. The use of multiple methodologies allowed to identify the optimal process conditions and the metabolic state that maximize the H_2 production from *C. celer*. Additionally, the tolerance towards substrate and metabolites accumulated during the fermentation was assessed to evaluate the robustness of *C. celer*. Based on this study, several conclusions can be drawn:

- The novel alkalithermophile *Caloramator celer* converts glucose to H_2, CO_2, acetate, formate, ethanol and traces of butyrate (Paper **I-IV**). YE and tryptone are used both as carbon and energy sources (Paper **I**). Occasional pyruvate overflow can occur under elevated growth rate (Paper **III**).
- End-product synthesis profiles, and consequently H_2 production, change in response to several modifications of the culture conditions (Paper **I-IV**). Supplementing the medium with YE (2 g/l), tryptone (2 g/l), $FeSO_4$ (200 mg/l), phosphate buffer (50 mM only in serum bottles) and glucose (10 g/l), and maintaining low H_2 concentration and acidic pH lead to H_2 yields above 3 mol H_2/mol glucose with the highest being 3.53 mol H_2/mol glucose (i.e. 88 % of the theoretical yield) (Paper **I**, **II**, **IV**).
- *C. celer* shows a great metabolic flexibility allowing redistribution of fluxes at key metabolic nodes to simultaneously control redox state and efficiently harvest energy from the substrate even under unfavorable conditions (e.g. low pH and/or high H_2 concentrations). The distribution of the fluxes at key metabolic nodes is found to be a function of thermodynamics as well as several physiological factors including genome content, growth and glycolytic rate, need for maintaining intracellular redox and pH homeostasis, and energy conservation strategies (Paper **IV**).
- The synthesis of formate and ethanol competes with H_2-evolving reactions for the disposal of reducing equivalents (Paper **I**, **II**, **IV**), but serves as an alternative to proton reduction for regulating the redox state when H_2ases are inhibited (Paper **IV**).
- The fate of carbon and electron flow at the pyruvate node significantly impacts the conversion efficiency to H_2. In order to maximize the H_2 yield pyruvate needs to be oxidatively decarboxylated via pyruvate:ferredoxin oxidoreductase

(Paper **IV**). However, the direct correlation observed between formate synthesis and anabolic activity (Paper **II**, **IV**) implies that under controlled conditions growth at high rates is detrimental for achieving high H_2 yield. This observation, together with the possible non-involvement of PPi in catabolic metabolism (Paper **VI**) and the electron flow mainly directed to H_2 at low growth rates or in stationary phase (Paper **II**, **IV**), indicates that in *C. celer* H_2 synthesis is not coupled with high anabolic activity. Furthermore, it suggests a possible role of PFL in anabolic metabolism (Paper **II**, **IV**).

- Cellular energetics plays a role in the distribution of carbon and electron flow. For example, increasing cell maintenance associated to non-growth reactions increases the overall H_2 yield (Paper **IV**).
- The lack of correlation between abundance of transcripts and end-product profile under culture perturbation experiments suggests that other factors besides gene expression are more prominently involved in the regulation of metabolic fluxes (Paper **III**).
- Kinetics of growth and H_2 production are inhibited, albeit to different degrees, whereas H_2 yields remain marginally affected even in presence of considerable concentrations of inhibitors (Paper **III**). Acetate, the main soluble metabolite of the fermentation, inhibits H_2 productivity due to the increased ionic strength in the medium, rather than the uncoupling effect of the undissociated form (Paper **III**). The critical substrate and salt concentrations estimated for *C. celer* suggest that this organism is not particularly osmotolerant (Paper **III**).
- Comparative genomics and functional prediction analysis identified genes encoding for putative enzymes involved in sugar transport, glycolysis, pyruvate catabolism and disposal of reductants, electron shuttling and energy conservation as well as putative genes belonging to the Rex regulon (Paper **VI**). Additionally, genomic data (Paper **V**, **VI**) provided valuable information for interpretation of experimental results, and for directing experimental design (Paper **III**, **IV**) and possibly future metabolic engineering strategies for improving H_2 production.

6. RECOMMENDATIONS FOR FURTHER RESEARCH

Although the quest for establishing an efficient and cost-effective fermentative H_2 production process is far from being complete, model organisms for thermophilic H_2 production (i.e. *Ca. saccharolyticus*, *T. maritima*, *Cl. thermocellum*, *P. furiosus* and *Cal. subterraneus* subsp. *tengcongensis*) have been studied in detail for more than a decade expanding the knowledge on their physiology and intricate metabolism. Conversely, this study has just scratched the surface of the mechanisms that control the metabolic flux distribution in *C. celer*. Nevertheless, it contributes to the ongoing investigation of the carbon and energy metabolism in thermophilic fermentative H_2-producing microbes. Several findings from this study merit further research in order to gain a better understanding of the metabolism of *C. celer* and to optimize the fermentative H_2 production process from this alkalithermophilic bacterium. The optimization of the medium composition and fermentation conditions could be further explored to enhance the yields and productivities by investigating other factors (e.g. minerals, vitamins, alternative carbon and nitrogen sources, temperature, cell immobilization and alternative fermentation systems) and/or by employing different methodological approaches (e.g. experimental design methods). In particular medium optimization should aim for a reduction of excess nutrients and consequently of the costs (Willquist and van Niel 2012, Kridelbaugh et al. 2013), whilst maintaining the fermentation performances unaltered.

This study suggests that operating under conditions that favor high rate of biomass synthesis harms the overall efficiency of the H_2 production process. However, in few occasions it was difficult to distinguish the effects of the high anabolic activity from those of other parameters under investigation. Performing chemostat cultivations instead of batch cultivations would allow to keep the growth rate as an independent variable and thus to better identify the causes of the metabolic behaviors observed in this study. Since in *C. celer* higher H_2 yields were obtained at low growth rates or during stationary phase when most of the substrate was used for cell maintenance it would be worth to explore different fermentation setups such as two-stage fermentation where rapid biomass synthesis with consequent high formate and ethanol production can be decoupled from the efficient hydrogenogenic phase (Kivistö et al. 2013b). Additionally, cell immobilization methods could be employed to enhance biomass retention and further increase H_2 productivities (Zhang et al. 2007, Koskinen et al. 2008b). Both approaches should be studied first separately and then combined.

Despite the fact that this study highlights some of the fermentation conditions and metabolic states that affect the distribution of the metabolic fluxes at key metabolic branch points, a deeper knowledge on the regulation mechanisms at transcriptional, translational, and post-transcriptional level is required. Key catabolic enzymes (e.g. GAPDH, PFL, PFOR and ALDH/ADH) should be the target of thorough kinetics studies to determine their allosteric regulation and how intracellular fluctuations of cofactors (e.g. NAD^+, $NADP^+$, NADH, NADPH, Acetyl-CoA, HS-CoA, AMP, ADP, ATP, PPi and other intermediates of the cellular catabolism) alter the enzymatic activity and consequently the metabolic fluxes in response to fermentation conditions. Further transcriptional and comparative genomic studies should aim to elucidate the regulatory role of the Rex protein in the expression of key genes of the central carbon metabolism in *C. celer* and to assess the impact of this transcriptional regulation system on the distribution of metabolic fluxes and end-product profile.

Given the osmosensitivity of *C. celer*, means should be explored to either make the strain less sensitive to high osmotic pressure or remove the end-products from the fermentation vessel. The former goal could be achieved by adaptive laboratory evolution (Dragosits and Mattanovich 2013) and genetic engineering (Nicolaou et al. 2010), while the latter by implementing technologies for removal of liquid end-products such as dialysis or cell-recycling (Holst et al. 1997).

Developing reliable genetic tools for *C. celer* would open a number of possibilities to improve the H_2 production efficiency and the robustness of this strain as well as to broaden its substrate utilization range. The most common strategy to improve the production yields of bioconversions involves the deletion of competing reactions (Lee et al. 2008, Hallenbeck and Ghosh 2012). This study reveals that both ethanol and formate production are important to maintain the redox state in check when H_2ases are inhibited. Therefore, it can be expected that knocking out either ADH or PFL might harm the robustness displayed by *C. celer* towards changes in H_2 concentration. Moreover, PFL is essential for growth at high rates. It might be instead wiser to heterologously express a FHL to convert formate to CO_2 and H_2. One of the main limitations of *C. celer* is the restricted number of substrates that can be metabolized. Genetic engineering should aim to introduce novel transporters and/or pathways enabling the consumption of pentose sugars and glycerol as well as hydrolases to breakdown lignocellulosic materials. Currently, only hexose- and/or protein-rich feedstocks could be used as substrate for the H_2 production from the wild-type strain. This limitation could also be overcome by employing *C. celer* in *de novo* co-cultures together with carefully selected partners. Assessing the possibility for *C. celer* to detoxify biomass hydrolysates by converting furan and aromatic aldehydes to less-toxic alcohols, as observed in closely related species, is also worth further studies.

7. REFERENCES

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