Caldicellulosiruptor Saccharolyticus: an Ideal Hydrogen Producer?

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Abstract

Caldicellulosiruptor saccharolyticus is an extremely thermophilic, strictly anaerobic, Gram-positive and cellulolytic microoranism with a natural ability to produce hydrogen (H_2) at nearly theoretical maximum yield, i.e. 4 mol/ mol of glucose. Due to its CO_2-free combustion and high energy density, among other desirable properties, H_2 is touted as a fuel of the future. Biological H_2 production by thermophilic microorganisms utilizing waste biomass holds a huge potential as the most environment friendly process for commercial H_2 production. For this reason, it is imperative to identify and develop a microorganism with the most beneficial properties as an ideal H_2 producer may possess.

During this work, the physiology and metabolism of *C. saccharolyticus* was studied in detail, which revealed that – i) it can sustain its growth in the absence of any means of removal of H_2 from the reactor, ii) it can utilize the sugars in wheat straw hydrolysate effectively for its growth and H_2 production with 67% conversion efficiency, iii) Methane can replace N_2 as a sparging gas for removal of H_2 from the culture, without affecting the growth and H_2 production by *C. saccharolyticus*, iv) by-products of its fermentative metabolism can be converted to methane by anaerobic digestion, v) it is capable of assimilating sulphate as a primary sulphur source, vi) can form biofilms when co-cultured with *C. owensensis*, which can be an effective means of retaining biomass in the reactor, and vii) Up-flow anaerobic (UA) reactor with granular sludge offer better alternative that a continuously stirred-tank reactor for improving its volumetric H_2 productivity (Q_{H2}).

Moreover, to improve the Q_{H2} further, the methods of evolutionary engineering were used to develop osmotolerant mutant strains of *C. saccharolyticus*: i) *C. saccharolyticus* G10, capable of growing in a medium containing up to 100 g/L of glucose, and ii) *C. saccharolyticus* AG6 capable of growing in a medium containing approx. 13.2 g/L of sodium aceate and 30 g/L of glucose. Indeed, the cultivation of one of the osmotolerant strains in an chemically optimized medium improved the Q_{H2}.

Furthermore, experimente were performed to understand the barriers to genetic modification of *C. saccharolyticus*. The studies revealed that the methylation of the foreign DNA by C5-cytosine-specific methyltransferase may help overcome the restriction-modification system of *C. saccharolyticus*. In addition, uracil-auxorophic strains of *C. saccharolyticus* were developed, which can be used as a host to perform genetic modifications.

In conclusion, the knowledge and newfound properties obtained in this study should be combined to create a strain of *C. saccharolyticus* that will fulfil nearly all the requirements to make it into an ideal H_2 producer.

Key words
Caldicellulosiruptor saccharolyticus, *Caldicellulosiruptor owensensis*, hydrogen, volumetric hydrogen productivity, biofilm, osmotolerance, wheat straw hydrolysate, evolutionary engineering, CSTR, UA reactor and uracil auxotrophy

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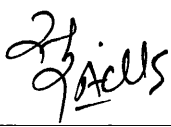

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Cover:

Scanning electron micrograph of *Caldicellulosiruptor* species

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To that only moment

When you **both** smiled to see me cry

Popular scientific summary

What is common between the science of physics and global politics? They both define power in terms of energy. Indeed, the uneven distribution of petroleum reserves has been one of the main reasons of social, political and economic problems worldwide. Even more worrying is the rapid increase in the levels of greenhouse gases in the atmosphere. This is mainly caused by our heavy use of petroleum based fuels as a source of energy. This has led to global warming. What if we switch to using a fuel which does not cause any carbon dioxide emissions? Do we know any such fuel? Can we produce it in a way that is safe for the environment?

The answer we are looking for is – Hydrogen gas. It is in many ways an ideal fuel, termed by many experts as "the fuel of the future". Indeed, it is a powerful and cleanest of the fuels producing nothing but water upon combustion. But, the current methods of producing hydrogen gas are harmful for the environment. These methods use petroleum based fuels for the production of hydrogen gas. Therefore, during this work, I studied a microorganism which can produce hydrogen gas using a method which is safer for the environment and does not depend on petroleum based fuels. The organism is - *Caldicellulosiruptor saccharolyticus* and the method is known as thermophilic biohydrogen production.

During this work, I confirmed a few known features of *Caldicellulosiruptor saccharolyticus* and also discovered some new features. As a hydrogen producer, *Caldicellulosiruptor saccharolyticus* has many good properties. It has tools to break down almost any kind of biological polymer to individual sugars. And also is able to consume these sugars to produce hydrogen gas with utmost efficiency possible. It does not stop producing hydrogen gas even after its niche is saturated with the gas. It does not need any expensive nutrients for its growth, which helps in reduction of costs of hydrogen gas production.

But, in its natural state *Caldicellulosiruptor saccharolyticus* is not perfect. It cannot grow in an environment with very high amounts of sugars. This is a major drawback for its industrial application. Therefore, I exposed the organism to stressful conditions to study its response. The organism responded by adapting to changing conditions to give its variant - *Caldicellulosiruptor saccharolyticus* G10, which is able

to grow in presence of high amounts of sugars. I also determined the suitable conditions which encourage the organism to form biofilm. Further studies showed that the biofilms of this organism can be helpful to increase its rate of producing hydrogen gas.

Also, genetic modification of *Caldicellulosiruptor saccharolyticus* has been a challenge for the researchers worldwide. I discovered that the problem lies in its defence mechanism. I also managed to create its variant - *Caldicellulosiruptor saccharolyticus* URA^-, which can be an efficient host to study its possible genetic modifications.

Altogether, the knowledge obtained in this study can lead to create a modified variant of *Caldicellulosiruptor saccharolyticus*. This variant will have almost all the features of an ideal hydrogen producer needed for industrial production of hydrogen gas.

Populärvetenskaplig sammanfattning

Vad har den fysikaliska vetenskapen gemensamt med global politik? De definierar båda kraft i termer av energi. Och faktiskt, den ojämna distributionen av oljetillgångar har varit en av huvudanledningarna till sociala, politiska och ekonomiska problem i världen. Än mer oroväckande är den snabba ökningen av växthusgaser i atmosfären. Detta är främst orsakat av vår höga användning av petroleumbaserade produkter som energikälla, vilket har lett till global uppvärmning. Tänk om vi kunde växla om till ett bränsle som inte skapar några koldioxidutsläpp? Känner vi till något sådant bränsle? Kan vi producera det på ett sätt som är säkert för miljön?

Svaret vi söker är – vätgas. Det är på många sätt ett idealiskt bränsle, beskrivet av många experter som ”framtidens bränsle”. Och ja, det är både kraftfullt och det renaste bränsle vi känner till då det endast bildar vattenånga vid förbränning. Men de nuvarande metoderna för vätgasframställning är skadliga för miljön eftersom de vid produktion använder sig av petroleumbaserade bränslen. Därför har jag i det här arbetet arbetat med studera en mikroorganism som kan producera vätgas med en metod som är både säkrare för miljön och inte är beroende av några petroleumbaserade bränslen. Organismen heter *Caldicellulosiruptor saccharolyticus* och metoden kallas termofilisk produktion av biovätgas.

I detta arbete bekräftade jag en del kända egenskaper hos *Caldicellulosiruptor saccharolyticus* och upptäckte även en del nya. *Caldicellulosiruptor saccharolyticus* har många goda egenskaper som vätgasproducent. Den har verktyg för att bryta ned nästan alla typer av biologiska polymerer till dess individuella sockerbeståndsdelar. Den är även ytterst effektiv på att konsumera dessa sockermolekyler med vätgas som produkt. Den slutar inte att producera vätgas även om dess omgivning är mättad av gasen. Den behöver inte några dyra näringsämnen för att kunna växa, vilket hjälper till att reducera kostnaden för vätgasproduktion.

Men *Caldicellulosiruptor saccharolyticus* är inte perfekt i sitt naturliga tillstånd. Den kan inte växa i en miljö med mycket höga halter av socker. Detta är en stor nackdel för dess industriella tillämpning. Därför utsatte jag organismen för stressande

betingelser och studerade dess reaktion. Organismen svarade med att anpassa sig till de förändrade faktorerna vilket gav upphov till dess variant - *Caldicellulosiruptor saccharolyticus* G10, som är förmögen att växa i närvaro höga sockerhalter. Jag bestämde även de lämpliga förutsättningarna för att främja organismens bildning av biofilm. Vidare studier visade att organismens biofilmer kan vara gynnande för en ökad vätgasproduktion.

Genetisk modifikation av *Caldicellulosiruptor saccharolyticus* har dessutom varit en utmaning för forskare värden över. Jag fann att problemet ligger i dess försvarsmekanism. Jag lyckades även skapa dess variant, *Caldicellulosiruptor saccharolyticus* URA^{-}, som kan vara en effektiv värd vid studier av dess möjliga genetiska modifikationer.

Sammantaget kan den erhållna kunskapen i denna studie leda till en modifierad variant av *Caldicellulosiruptor saccharolyticus*. Denna variant kommer att inneha nästan alla de egenskaper som krävs av en idealisk vätgasproducent för industriell framställning av vätgas.

(Translated from the original text in English)

Courtesy: **Oscar Hallbäck**

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Reassessment of hydrogen tolerance in *Caldicellulosiruptor saccharolyticus.***

Willquist K., Pawar S. S. and van Niel E. W. J. Microbial Cell Factories 10:111 (2011)

II. **Biohydrogen production from wheat straw hydrolysate using *Caldicellulosiruptor saccharolyticus* followed by biogas production in a two-step uncoupled process.**

Pawar S. S., Nkemka V. N., Zeidan A. A., Murto M., and van Niel E. W. J. International Journal of Hydrogen Energy, 38(22), 9121-9130 (2013).

III. **Evaluation of assimilatory sulphur metabolism in *Caldicellulosiruptor saccharolyticus*.**

Pawar S. S. and van Niel E. W.J. Bioresource Technology, 169(0), 677-685.

IV. **Biofilm formation by designed co-cultures of *Caldicellulosiruptor* species as a means to improve hydrogen productivity**

Pawar S. S., Vongkumpeang T., Grey C., and van Niel E. W. J. *Submitted*

V. **Development of osmotolerant strains of *Caldicellulosiruptor saccharolyticus* as a means to improve hydrogen productivity**

Pawar S. S., Zurawski J. V., Lahiry A., Blumer-Schuette S. E., Kelly R. M., and van Niel E. W. J. *Manuscript*

VI. Genetic modification of *Caldicellulosiruptor saccharolyticus*: Elucidation of its restriction-modification system and development of a toolkit

Pawar S. S., Byrne E., Muñoz de las Heras A., van Niel E. W. J., and Rådström P. *Manuscript*

A part of the summary of this thesis is adopted and modified (when necessary) from the following review article, as the concerned part was –i) exclusively written by the author of this thesis, ii) originally intended for the summary of this thesis but had to be published in advance (see also 'My contributions to the studies'):

VII. Thermophilic biohydrogen production: how far are we?

Pawar S. S. and van Niel E. W. J. Applied Microbiology and Biotechnology, 97(18), 7999-8009 (2013).

I have also contributed to the following articles:

A. Assessment of metabolic flux distribution in the thermophilic hydrogen producer *Caloramator celer* as affected by external pH and hydrogen partial pressure

Ciranna A., Pawar S. S., Santala V., Karp M., and van Niel E. W. J. Microbial Cell Factories, 13(1), 48 (2014).

B. Design of a novel biohythane process with high H_2 and CH_4 production rates.

Willquist K., Nkemka V. N., Svensson, H., Pawar S. S., Ljunggren M., Karlsson H., Murto M., Hulteberg C., van Niel E. W. J., and Liden, G. International Journal of Hydrogen Energy, 37(23), 17749-17762 (2012).

My contributions to the studies

I. I planned and performed continuous cultures, enzyme kinetics and its modelling, and metabolite analysis under the guidance of Dr. Karin Willquist. I drafted the manuscript along with Dr. Karin Willquist.

II. I planned the experiments related to dark fermentation with the help of Dr. Ahmad A. Zeidan. I performed all the experiments related to dark fermentation. I drafted the manuscript except for the part related to biogas production.

III. I conceived the idea and planned the contents of the article. I planned and performed all the experiments and drafted the entire manuscript.

IV. I conceived the idea and planned the contents of the article. I planned all the experiments. I performed most of the experiments. I drafted the entire manuscript.

V. I conceived the idea and planned the contents of the article. I planned all the experiments. I performed most of the experiments except for microarray assays. I drafted the entire manuscript.

VI. I conceived the idea and planned the contents of the article. I planned all the experiments. I trained a couple of master students – Alejandro and Eoin, who performed most of the experiments under my supervision. I drafted the entire manuscript.

VII. I and Dr. Ed WJ van Niel planned the contents of the manuscript together. The parts I wrote of this article, are covered by the following sub-sections: Introduction, Pure culture studies, Enzymes of thermophilic hydrogen production, Complex media, Appropriate feedstocks and LCA/economical feasibility studies inherent on process development. I prepared the figure and wrote the text summarizing properties an ideal hydrogen producer may possess. I prepared all the tables.

Abbreviations

ATP	Adenosine triphosphate
NAD^+	Nicotinamide adenine dinucleotide, oxidised form
NADH	Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
Fd_{red}	Reduced ferredoxin
Fd_{oxd}	Oxidized ferredoxin
SSF	Simultaneous saccharification and fermentation
ABC	ATP binding cassette
PTS	Phosphotransferase system
D	Dilution rate
Q_{H2}	Volumetric hydrogen productivity
CSTR	Continuously stirred tank-reactor
UA	Up-flow anaerobic
STP	Standard temperature and pressure
MGE	Mobile genetic elements
P_{H2}	Hydrogen partial pressure

Contents

1. Hydrogen

Elemental hydrogen is the simplest and the most abundant element in the known universe (Lubitz & Tumas, 2007). However, due to its reactive nature, most of it is bound in molecules such as, water or most organic compounds. Naturally, these compounds are also the main sources of molecular hydrogen (H_2). Due to its low volumetric mass density, H_2 is one of the rarest of the gases present in the atmosphere at ground level, i.e. 0.6 ppm (Glueckauf & Kitt, 1957). Thus, H_2 needs to be produced artificially for its various applications.

1.1. Applications of H_2

Although H_2 is mostly inert at standard conditions (STP), under appropriate conditions it can react with many elements to form useful compounds with various applications. It is also a highly flammable gas with the ability to burn in air at a concentration ranging from 4-75% v/v (Carcassi & Fineschi, 2005) with -284 KJ/mol of combustion enthalpy producing only water. These inherent properties among others allow it to be used in various applications as listed below.

1.1.1. H_2 as a fuel

Our heavy dependency on fossil energy sources has created societal problems related to its inherent environmental pollution. In addition, the accumulation of greenhouse gases has been one of the primary reasons of many climatic changes around the globe. It urges for alternative, clean and renewable sources, but despite fuelling huge research interests among scientists, there is hardly any tangible success so far. The utopian world of energy sufficiency, without any hazardous emissions - is thought to be plausible by many, if only renewable H_2 could replace fossil-based energy carriers (Schrope, 2001). As early as 1874 Jules Verne fancied the concept of a 'Hydrogen economy' in his book -'The Mysterious Island' (Hoffmann, 2001). Indeed, as a fuel H_2 has many desirable properties (Table 1), among others, rapid

flame velocity, emissions free of greenhouse gases, higher energy yield by weight, low minimum ignition point and very high (research) octane number (Balat & Kirtay, 2010; Ingersoll, 1996; Luque et al., 2011; Sequeira & Santos, 2010). It's not a surprise that H_2 is termed as a fuel of the future. Interestingly, the arrival of fuel-cell electric vehicles (FCEV) on the market has provided another alternative to electric cars for reducing pollution in densely populated cities.

Table 1 Technical properties of Hydrogen in comparison with other fuels. (data taken from (Altun et al., 2013; Sequeira & Santos, 2010))

Properties	Ethanol*	Petroleum	Methane	Hydrogen
Boiling point, *K*	351	350-400	111.7	20.3
Gas density, $kg \cdot m^{-3}$	-	4.68	0.66	0.08
Liquid density, $kg \cdot m^{-3}$	789	702	425	71
Auto-ignition in air, *°C*	368	222	534	585
Ignition energy, *MJ*	-	0.25	0.3	0.02
Flame velocity, $cm \cdot S^{-1}$	-	30	34	270
LHV, $MJ \cdot kg^{-1}$	28.8	44.38	50	120

*Data represents values for fuel ethanol

1.1.2. Hydrogen as a commodity

Apart from being an ideal fuel, H_2 also has a number of applications in various industries (Table 2). Currently, of all the H_2 produced, 54% is used in synthesis of ammonia, 35% in petro-chemical industry, 6% in electronic industry, 3% in metal and glass industry, and the remaining 2% in the food industry (Chaubey et al., 2013). Ammonia is mainly used for production of fertilizers for agriculture, whereas, desulphurization is very essential for obtaining cleaner gasoline from crude oil. In metallurgy, it is very important to create a reducing environment for the extraction of various metals (Table 2). Thus, H_2 has a huge impact on our daily lives through direct or indirect means.

Table 2 Applications of H_2 as a non-fuel commodity. (modified from (Sequeira & Santos, 2010))

Industry	Uses
Petroleum	Desulphurisation and hydrocracking
Chemical	Production of ammonia, methanol, hydrogen peroxide and in several other hydrogenation reactions
Food	Conversions of sugars to poly-ols (sweeteners), hydrogenation of edible oil
Plastics	Production of nylons, polyurethanes, polyesters, cracking of used plastics
Metals	In the production of metals e.g. iron, nickel, molybdenum, magnesium, and various other metallurgical applications
Electronics	Manufacturing of poly-silicon, vacuum tubes, light bulbs
Glass	Float-glass process, glass polishing, production of fibre-glass
Electricity	Coolant for generators and motors, nuclear fuel processing

1.2. Methods of Hydrogen production

Presently, about 54 million tonnes of H_2 is produced worldwide per year, of which 95% is captive i.e. produced and used on-site, whereas about 3 million tonnes is sold (merchant H_2) to other industries (Mueller-Langer et al., 2007). Owing to factors such as, decreasing quality of crude-oil or increased demand of food (and thus fertilizers), the global demand of merchant H_2 is set to increase by about 6-7 million tonnes through year 2013 (Freedonia, 2010). The very first documented instance of large-scale H_2 production dates back to the end of 18^{th} century (Langins, 1983). At present, there are plenty of different methods by which H_2 can be produced (Figure 1), however, very few of them are currently used industrially.

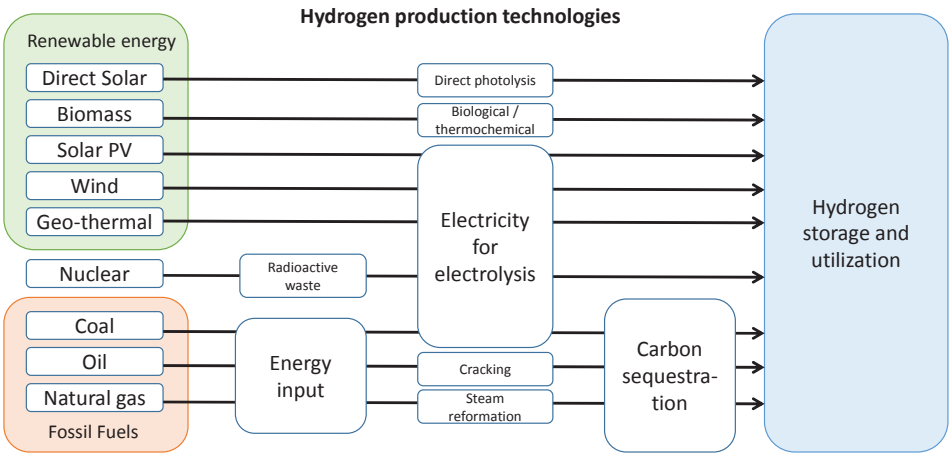

Figure. 1 An overview of H_2 production methods[1]

1.2.1. Current methods of hydrogen production

Currently, there are four established industrial H_2 production processes – i) steam methane reformation (SMR), ii) partial oxidation, iii) auto-thermal reformation and, iv) steam-iron process (Chaubey et al., 2013). SMR is by far the most cost-effective and well established process contributing to 40-50% of commercially produced H_2 from natural gas, coal or crude oil (Balat, 2008). The second most widely used process i.e. the process of partial oxidation involves heating of natural gas or other hydrocarbon in the presence of limited amount of oxygen (Steinberg & Cheng, 1989). The process of auto-thermal reformation is basically a combination of steam reformation with the process of partial oxidation (Chaubey et al., 2013). Lastly, the steam-iron process is a cyclic water-splitting process which involves coal, oil or biomass as an energy source and iron oxides (Hacker et al., 2000).

From a techno-economical perspective steam-reformation is currently the best method to produce H_2 (Mueller-Langer et al., 2007). However, about 96% of the H_2 produced at present is derived from all the afore-mentioned processes, which are entirely dependent on fossil fuels (Balat, 2008). Thus, it is imperative to develop

[1]Modified from http://www.fsec.ucf.edu/en/consumer/hydrogen/basics/production.htm

more eco-friendly, sustainable yet economically feasible methods utilizing renewable sources for H_2 production.

1.2.2. Sustainable methods of hydrogen production

The sustainable methods of H_2 production can be further divided into biological and non-biological methods (Figure 1). The non-biological methods mainly involve the application of existing renewable energy sources, such as, solar, wind or geothermal to perform electrolytic splitting of water to produce H_2 (Jacobson & Delucchi, 2011; Turner et al., 2008). However, further research and development is needed before a complete potential of these methods can be tested at a large scale (Turner et al., 2008).

Alternatively, biological means of H_2 production may also provide viable alternatives. Biological hydrogen production mainly involves - i) Biophotolysis, ii) microbial electrolysis cells (MEC), and iii) (photo)fermentation (Lee et al., 2010). In general terms, biophotolysis is a photosynthetic process wherein light energy drives the machinery of H_2 generation from water splitting (Hallenbeck & Benemann, 2002). The MEC uses a combination of bacterial metabolism with electrochemistry to produce H_2 (Liu et al., 2005).

Fermentative H_2 production can be performed with the aid of light energy i.e. photofermentation or not i.e. dark fermentation. Photo- fermentation is a process wherein, photosynthetic bacteria use light energy to produce H_2 with the help of nitrogenase enzyme (Lee et al., 2010). Conversely, in dark fermentation microorganisms obtain energy from fermenting organic substrates for H_2 synthesis catalysed by hydrogenase. Microorganisms performing dark fermentation can utilize a variety of renewable biomass, such as, municipal, agricultural or forest waste, making dark fermentation even more eco-friendly (Chaubey et al., 2013).

Nonetheless, all these methods of biological H_2 production have their own advantages and disadvantages, all needing further research before their commercial realization is possible (Hallenbeck et al., 2012; Hallenbeck & Benemann, 2002; Kengen et al., 2009; Lee et al., 2010; Nath & Das, 2004).

The work described herein is mainly focused on the challenges and outcomes of the research pertaining to dark fermentation performed at elevated temperature (~70°C) using renewable biomass i.e. thermophilic biohydrogen production.

2. Thermophilic biohydrogen production

The process of H_2 production using biological material such as, municipal, agricultural or forest waste, at elevated temperature by thermophilic microorganisms is termed as 'thermophilic biohydrogen production'. Clearly, the choice of appropriate microorganism(s) is central to its development. Moreover, the choice of microorganism may also influence the choice of an appropriate feedstock and culture conditions for optimum H_2 production. Thus, it is significant to understand the basic physiological properties of H_2 producing thermophilic microorganisms[2].

2.1. Thermophilic vs mesophilic H_2 producers

Higher temperatures (≥ 60°C) are energetically more favourable for biological H_2 production (Stams, 1994), enabling thermophiles to reach higher H_2 yields than mesophiles ((Schönheit & Schäfer, 1995), *Property A*). As a consequence, thermophiles produce fewer by-products, i.e. especially acetic acid as its generation is accompanied with formation of an extra ATP (Kengen et al., 2009). Moreover, thermophilic conditions seem to restrict contamination by hydrogenotrophic methanogens. In general, thermophilic H_2 producers have also higher H_2 tolerance, however, the latter may vary depending on the sugar(s) present in the feedstock (**Paper I**). Even so, a drawback of thermophiles is their relatively low volumetric productivity, as they have a tendency to grow in lower cell densities in suspension cultures than mesophiles (Chou et al., 2008). Highest biological H_2 productivities

[2] Hereafter, the desirable physiological properties in an ideal H_2 producer will be identified in brackets as '*Property A to I*'.

ever reported have been for mesophilic cultures, but their accompanying low H_2 yields remain a critical problem (Das, 2009).

2.2. Microorganisms of thermophilic H_2

Thermophilic H_2 producers are found within both the bacterial and the archaeal domain, and several of them have been characterized with their genome annotated (see list in (VanFossen et al., 2008)). Most of them are able to hydrolyze various polysaccharides (Blumer-Schuette et al., 2008) and can ferment the released hexoses and pentoses to H_2 with yields close to the theoretical maximum (or Thauer limit, 4 mol H_2/mol hexose (Thauer et al., 1977)). Recent reviews list many of those microorganisms involved (Kengen et al., 2009; van Niel et al., 2011), so here only a selection of the best performers is given (Table 3 and 4). Most of these organisms were isolated from extremely hot and reducing conditions (Fiala & Stetter, 1986; Huber et al., 1986; Mäkinen et al., 2009; Rainey et al., 1994; Xue et al., 2001), under which they produce reduced metabolic end-products, including H_2, as an electron sink for reducing equivalents (Thauer et al., 1977).

Table 3 Overview of thermophilic hydrogen producing microorganisms (continued from (Kengen et al., 2009))

Organism	Domain	T_{opt} (°C)	Mode	Substrate	Y_{H2}	References
Caloramator celer	Bacteria	67	Batch	Glucose	3.36	(Ciranna et al., 2011)
Clostridium stercorarium DSM 2910	Bacteria	58	Continuous	Lactose	1.57	(Collet et al., 2004)
Thermovorax subterraneus	Bacteria	70	Batch	Glucose	1.4	(Mäkinen et al., 2009)

Under stressful conditions of high H_2 partial pressures (P_{H2}) and/or high medium osmolality - organisms tend to shift their metabolism to other reduced end-products

such as lactate, ethanol and alanine (Table 2, (Kengen et al., 2009; Willquist et al., 2009) and **Paper I**), which in turn negatively affect the H_2 yield. Although when purified, these by-products may have a reasonable market value for a variety of purposes, but, their concentration in the effluent will be too low for an economically viable downstream process. Alternatively, microorganisms can be engineered to produce more valuable product under stress (*Property G*). On the other hand, the by-products can be fed to a complementary process, which can further produce valuable products including H_2 and methane (Hallenbeck & Ghosh, 2009).

2.3. Enzymes of thermophilic H_2

2.3.1. The hydrogenases

Evolution of H_2 through reduction of a proton is carried out by metalloenzymes, i.e. hydrogenases, which differ with respect to their size, structure, electron donors and metal ions present in their active site (Meyer, 2007; Vignais & Billoud, 2007). Hydrogenases found in thermophilic microorganisms can be classified based on the metal ions present in their active site, viz., [FeFe]-, [NiFe]- and [Fe]-hydrogenases (Meyer, 2007). [NiFe]-hydrogenases have been reported in both bacteria and archaea. However, [FeFe]-hydrogenase have only been reported in anaerobic bacteria (Meyer, 2007; Vignais & Billoud, 2007). Whereas, the [Fe]-hydrogenases (Hmd hydrogenases) have been reported only in a few methanogenic archaea (Meyer, 2007). Hydrogenases are known to be very sensitive to oxygen (O_2) (Vignais & Billoud, 2007); even 1% of O_2 can completely inhibit their H_2 forming capacity but not H_2-oxidation (Lukey et al., 2011). Hallenbeck and co-authors argue that, for a variety of reasons, a limited amount of aerobic respiration along with fermentation may help achieve H_2 yields near the absolute maximum (12 mol/mol of hexose) through complete conversion of glucose to CO_2 via the TCA cycle (Hallenbeck et al., 2012; Hallenbeck & Benemann, 2002). A recent study revealed successful engineering of an O_2-tolerant [NiFe]-hydrogenase, through site-directed mutagenesis (Lukey et al., 2011). In addition, native O_2-tolerant hydrogenases have been found in *Ralstonia eutropha* H16 (Burgdorf et al., 2005) and *Aquifex aeolicus* (Guiral et al., 2006). Such O_2-tolerant hydrogenases could be instrumental in performing micro-aerobic fermentations (*Property I*). However, further research is needed to assess the validity of this hypothesis.

Table 4 Metabolic features of thermophilic hydrogen producers (adopted from (Pawar & van Niel ,2013))

Organism	Fermentability of feedstocks / polymers	CCR*	Auxotrophy§	Electron carriers	Hydrogenase‡	Reductant Sink	References
Clostridia (Cl. thermocellum)	starch, cellulose, lignocellulose	Yes	No	NADH, Ferredoxin	Uptake, Fe-only, FNOR	alcohol, acids, lactate	(Johnson et al. 1981; Desvaux 2006)
Thermococcales (Pyroccus furiosus)	maltose, cellobiose, β-glucans, starch	No	Yes	Ferredoxin	MBH, NiFe-only, FNOR	alanine, ethanol	(Hoaki et al. 1994; Maeder et al. 1999; Silva et al. 2000; Robb et al. 2001)
Thermotogales (T. maritima / T. neapolitana)	Cellulose, xylan, Starch, cellobiose, Lignocellulose	Yes	No	NADH, Ferredoxin	Fe-only, NMOR, FNOR	lactate, alanine	(Schönheit et al. 1995; Vargas et al. 1996; Rinker et al. 2000; Bonch-Osmolovskaya 2001)
Caldicellulosiruptor (C. saccharolyticus)	Cellulose (avicel, amorp.), xylan, Pectin, α- and β-glucan, Lignocellulose, Guargum	No	No	NADH, Ferredoxin	Fe-only, NiFe-only	lactate, ethanol	(Rainey et al. 1994; de Vrije et al. 2007; van de Werken et al. 2008; Ivanova et al. 2009; Willquist et al. 2012)
Thermoanaerobacter (T. tengcongensis MB4)	Starch, sucrose, glycerol	Yes	Yes	NADH, Ferredoxin	Fe-only, NiFe-only	ethanol	(Xue et al. 2001; Warner et al. 2003; Soboh et al. 2004)

*CCR, carbon catabolite repression; §Auxotrophy to amino acids; ‡Types of *hydrogenases* – uptake, NiFe type H_2 uptake hydrogenase, FNOR, Ferredoxin:NAD(P)H oxidoreductase, Fe-only, Fe-only evolution hydrogenase, NiFe-only, NiFe-only evolution hydrogenase, NMOR, NADH:methylviologen oxidoreductase and MBH, membrane-bound hydrogenase

Hydrogenases use NADH or reduced ferredoxin (Fd_{red}) as electron donors, which are formed in the catabolism of organic substrates (Kengen et al., 2009). Under standard conditions, the mid-point redox potential for redox couples, NAD^+/NADH and oxidized ferredoxin (Fd_{ox})/ Fd_{red} is -320 mV and -398 mV, respectively (Thauer et al., 1977), whereas its value for H_2 is -414 mV (Angenent et al., 2004), which clearly indicates that ferredoxin (Fd)-dependent H_2 production is thermodynamically more favourable (*Property C*). Alternatively, other relatively uncommon hydrogenases, such as ferredoxin:NAD(P)H oxidoreductase (FNOR, or electron-bifurcating hydrogenases) and membrane-bound hydrogenases (MBH) can also be desired for an ideal H_2 producer. FNOR produces H_2 using both - NADH and Fd_{red} simultaneously, by coupling unfavourable oxidation of NADH with exergonic oxidation of Fd_{red} (Schut & Adams, 2009), whereas MBH conserves valuable energy by coupling H_2 evolution to ATP synthesis via proton translocation (Sapra et al., 2003).

2.3.2. Other redox enzymes

The central carbon metabolism of thermophilic H_2 producers has diverse metabolic pathways to reduce electron carriers, i.e. Fd or NAD+. Bacterial H_2 producers oxidize glyceraldehyde-3-phosphate (GAP) via GAP dehydrogenase (GAPDH) generating 1 ATP and 1 NADH in the reaction. However, re-oxidation of the latter to H_2 is inherent to a thermodynamic constraint and thus instead it might easily be oxidized to undesired electron sinks, such as lactate and/or ethanol (for details see (Bielen et al., 2013)). In contrast, archaeal H_2 producers have a unique enzyme - GAP oxidoreductase (GAPOR), which oxidizes GAP generating 1 Fd_{red} but no ATP (Verhaart et al., 2010). Thus, introduction of GAPOR in bacterial H_2 producers may redirect more pyruvate flux towards acetate generating the required ATP and will consequently improve H_2 yields. In addition, a host of other redox enzymes may also be involved in oxidation of substrates other than conventional sugars such as, glycerol or rhamnose.

Similarly, at the pyruvate node, most of the distinguished thermophilic H_2 producers possess pyruvate:ferredoxin oxidoreductase (PFOR), which oxidizes pyruvate to generate Fd_{red} (Carere et al., 2012). In contrast, most mesophilic H_2 producers possess pyruvate:formate lyase (PFL) which generates formate (Carere et al., 2012). Some mesophilic organisms containing PFL also possess formate:hydrogen lyase (FHL) to oxidize formate to CO_2 and H_2. Nevertheless, PFOR remains a better

enzyme for oxidation of pyruvate, contributing to higher H_2 yields in thermophilic H_2 producers.

2.4. Growth medium and Feedstocks

Complex substrates, such as, yeast extract or peptone are regularly used as nutritional supplements to aid the growth of microorganisms at lab scale. Apart from providing amino acids, these medium supplements provide buffering capacity, reducing agents and chelators for metal ions. So far, most of the physiological studies on thermophilic H_2 producers have been performed containing such complex substrates (Kengen et al., 2009; van Niel et al., 2011). Moreover, some of the applied studies for more practical evaluation of biohydrogen production have also been performed using such substrates (Table 5). However, use of yeast extract and/or peptone can incur significant production cost in any industrial process (Ljunggren & Zacchi, 2010). Hence, an organism with the ability to synthesize all the amino acids will allow omission of complex substrates from the medium and thus help reduce the costs (*Property D*).

Over the years a variety of readily available feedstocks including industrial and municipal waste streams, glycerol from biodiesel production and various lignocellulosic materials have been evaluated for H_2 production (Table 5) with reasonable success. Lignocellulosic materials generally consist of a range of crop residues, dedicated energy crops, saw dust, forest residues and solid animal waste. It is often difficult to estimate the extent of future usage of these feedstocks due to their heterogeneous nature, uncertainties in their availability and sustainable recoverability, and their competing traditional applications (Gregg & Smith, 2010; Rosillo-Calle & Woods, 2012). Nevertheless, crop residues are estimated to be about 1010 tons/year globally (Lal, 2005) and hence are increasingly considered as a potential feedstock for biological H_2 production.

Lignocellulosic feedstocks largely contain lignin, hemicellulose and cellulose; albeit in diverse fractions depending on the nature of the feedstock (Sun & Cheng, 2002). Various physico-chemical methods are available to separate lignin from hemicellulose and cellulose. Solubilized polymers of hemicellulose and cellulose are further hydrolysed to mono-, or di-saccharides depending on the enzymes used for hydrolysis (Sun & Cheng, 2002). Application of thermophilic, hydrolytic enzymes will allow integration of hydrolysis and fermentation together in a single step, i.e. simultaneous saccharification and fermentation (SSF). *C. saccharolyticus*, *Clostridium*

thermocellum and *Thermotogales* are known to secrete hydrolytic enzymes required for hydrolysis of pre-treated lignocellulosic materials into monosaccharides (Table 4; *Property E*), which, therefore, become ideal candidates for such a consolidated process. Alternatively, hydrolytic enzymes produced by these organisms during fermentations can be separated from the effluent and used for the hydrolysis, minimizing the cost for enzymes. However, the cost of separation and re-usability of 'spent' effluent remains to be evaluated to conclude its feasibility.

A diverse fraction of monosaccharides, such as, glucose, xylose, arabinose, mannose, galactose and uronic acid are obtained upon hydrolysis of pre-treated lignocellulosic materials (Maris et al., 2006). Organisms having a diverse catabolic range for sugars will strengthen the robustness of the process and allows flexibility in the choice of feedstock. This will be of particular importance considering the seasonal and unpredictable availability of agricultural residues. Moreover, co-utilization of sugars present in the hydrolysate is very important for economically viable process. Thus organisms having a natural ability to co-utilize the sugars will ideally be preferred (*Property F*) over organisms unable to do so owing to 'carbon catabolite repression'.

2.5. The process

According to one of the twelve principles of green chemistry, "synthetic methods should be designed to maximize the incorporation of all materials used in the process into final product" (Anastas & Eghbali, 2010). As stated above, the thermophilic biohydrogen production can retrieve only up to 33% (4 mol H_2/ mol hexose) of the energy present in the substrate, with the remainder of it trapped in the by-products, such as acetate and/or lactate. Hence, to make thermophilic biohydrogen production environmentally friendly and economical; it needs to be coupled with another step capable of converting its by-products into either H_2 or any other useful product. Currently, only two alternatives are available, which can convert the by-products of thermophilic biohydrogen production to H_2: i) photo-fermentation, and ii) electro-hydrogenesis (Hallenbeck, 2009).

Photo-fermentation involves, conversion of organic acids to hydrogen by purple photosynthetic bacteria using the energy captured from sunlight. The potential of a two-stage process involving dark fermentation followed by photo-fermentation was evaluated in a European framework project – HYVOLUTION (Claassen et al., 2010). The project reported the overall efficiency of 57% (6.9 mol H_2/mol of hexose) – highest ever for microbial H_2 production (Özgür et al., 2010). However,

Table 5 Selection of thermophilic H_2 production using mixed/pure culture in various reactor types and/or industrial media. First four are best cases using model substrates (modified from Pawar & van Niel (2013)). *EGSB, Expanded granular sludge blanket; **HRT, hydraulic retention time, ¥, C - pH controlled, and nC - pH not controlled; ND - not determined.

Reactor type	Conditions				Feedstock / substrate	Organism / Source of inoculum for mixed culture	Enrichment with complex substrate	H_2 yield		Q_{H2} (mM/h)	Reference
	Method of Cultivation	HRT** (h)	T (°C)	pH (C/nC)¥				ml-H_2/gVS	mol/C6 mol		
CSTR	Continuous	20	70	6.7 (C)	Glucose	*C. saccharolyticus* G5	-	ND	3.0	20.7	**Paper V**
CSTR+carrier	Continuous	3	58	6 (C)	Glucose	Geothermal spring, Hveravellir, Iceland	Yeast extract	ND	1.54	45.8	(Koskinen et al. 2008)
Gas lift fermentor	Continuous	5	85	6 (C)	Pyruvate	*Thermococcus kodakaraensis* KOD1	Peptone	ND	2.18†	9.5	(Kanai et al. 2005)
UASB	Continuous	0.75	60	5 (C)	Sucrose	*Thermoanaerobacterium thermosaccharolyticum* PSU-2	Peptone	ND	1.3	152	(O-Thong et al, 2008)
UASB	Continuous	0.75	70	6.5 (C)	Glucose	*C. saccharolyticus* + *C. owensensis*	Yeast extract	ND	2.5	20	**Paper IV**
CSTR	Continuous	24	70	7 (nC)	Pig slurry	raw pig slurry	-	ND	ND	4.6	(Kotsopoulos et al. 2009)
CSTR	Continuous	24	55	5.25 (nC)	Rapeseed straw stillage from ethanol plant	thermophilic anaerobic digested manure from biogas plant	-	40.00	ND	6	(Luo et al. 2011)
CSTR	Continuous	12	60	6.8 (C)	Sugar factory waste water	sludge compost	-	ND	2.5	8.3	(Ueno et al. 1996)
CSTR	Continuous	4	60	5.5 (C)	Tofu waste water + glucose	hydrogenogenic labscale CSTR	-	ND	2.3	20.7	(Kim et al. 2010)
biofilm	Continuous	3	55	5 (C)	Sucrose	hydrogenogenic labscale CSTR	Yeast extract	ND	1.59	4.7	(Keskin et al. 2011)
Anaerobic filter	Continuous	24	70	5.4 (nC)	Wheat straw hydrolysate	enriched hydrogenogenic culture in CSTR	Yeast extract	ND	ND	0.9	(Kongjan et al. 2010)
Membrane bioreactor	Continuous	4	60	5.5 (C)	Tofu waste water	municipal sewage	-	ND	1.45	34	(Kim et al. 2011)
Upflow anaerobic	Continuous	2	55	5.5 (C)	Rice winery wastewater	municipal sewage	-	ND	1.9	3.9	(Yu et al. 2002)
Semi-continuous	Continuous	16	60	5.5 (nC)	Cassava stillage	UASB treating cassava stillage	-	56.70	ND	6.2	(Luo et al. 2010)
UASB	Continuous	24	55	nd	De-sugared molasses	anaerobic digested manure	Yeast extract	159.60	ND	7.8	(Kongjan et al. 2011)
EGSB*	Continuous	6	70	nd	Glucose, arabinose	household solid waste	-	ND	ND	4.7	(Abreu et al. 2010)
ASBR	Batch	96	60	5.5 (C)	palm oil mill effluent (POME)	palm oil mill wastewater treatment plant	Peptone	ND	2.60	1	(O-Thong et al. 2008)
ASBR	Batch	48	60	5.5 (C)	POME	palm oil mill wastewater treatment plant	Peptone	ND	ND	17	(Prasertsan et al. 2009)

the photo-fermentation was found to be the most expensive part of the process, accounting for more than 80% of the total cost (Claassen et al., 2010). Similarly, a promising, novel and elegant process of electro-hydrogenesis, which involves conversion of organic acids to H_2 using microbial fuel cells, has also been studied extensively (Liu et al., 2005). However, owing to a number of technical issues, its practical potential still remains to be proven (Hallenbeck, 2009).

Alternatively, the effluent collected from dark fermentation can also be treated in an anaerobic digester to produce methane (CH_4). During this study, a two-stage un-coupled process termed as '*biohythane*' was evaluated, wherein wheat straw hydrolysate was fermented by *C. saccharolyticus* to produce H_2 followed by the treatment of its effluent to produce CH_4 (**Paper II**). The overall process was able to convert about 57% of the energy contained in wheat straw to H_2 and CH_4 (**Paper II**). A detailed study focused on the various individual aspects of '*biohythane*' process highlighted many challenges, such as low H_2 productivity, the cost of nutrients and gas sparging for it to be practically applicable (Willquist et al., 2012).

2.6. Feasibility studies

So far, hardly any attempts have been made to evaluate the potential of any existing thermophilic biohydrogen production technology on a scale beyond that of laboratory studies. Nevertheless, a few techno-economic and LCA analyses have been performed using available literature to identify potential bottlenecks from environmental as well as techno-economical perspectives and steer the research towards pre-emptive measures.

2.6.1. Life cycle analysis

LCA involves assessment of environmental impact of different stages of a product's life cycle typically from cradle-to-grave. Ochs and co-workers performed a LCA evaluation (cradle-to-gate) of a proposed plant for thermophilic production of biohydrogen using potato steam peels under the assumption of a complete substrate

oxidation to produce only CO_2 and sewage as by-products[3] (Ochs et al., 2010). The study revealed that, during thermophilic fermentation, process inputs such as, phosphates and alkali – produced using fossil fuels, are the most potential contributors to high environmental impact[4]. Moreover, as discussed earlier, the presence of excessive salts in the growth medium can restrict the recirculation of process water, which can add to the environmental impact. Hence, measures need to be taken to minimize the usage of phosphate buffers in the growth medium as well as evaluating strategies for minimizing addition of alkali agents during fermentations. In addition, to minimize environmental impact, a complementary process capable of converting the generated by-products present in the effluent, thereby reducing the chemical oxygen demand, is an absolute requirement. For instance, a recent study reports about 93% reduction in COD after converting the effluent to methane via anaerobic digestion (Willquist et al., 2012).

2.6.2. Techno-economic evaluation

Techno-economic analysis assesses the technical feasibility of the different parts involved in the process and also the effect of different parameters on the cost of production with the help of computer programs such as Aspen Plus (Aspen Technology, Burlington, USA). Recent technological advancements allow heat recovery in the fermentation step. Such being the case, when compared to mesophilic fermentation, additional heat demand required in thermophilic fermentation did not incur significantly higher costs (Ljunggren & Zacchi, 2010). On the other hand, the production cost is largely influenced by – i) the cost of media ingredients, and ii) low substrate (sugar) concentrations (Ljunggren & Zacchi, 2010). As discussed above, yeast extract (or similar) is the most expensive component of the medium and is not needed by the H_2 producers having the ability to synthesize most of the growth factors present in the yeast extract. Secondly, low substrate concentrations in the medium will require larger reactors along with larger facility and consequently will demand more water and energy. Increasing the substrate concentration may not be a quick and easy solution; as it increases the osmolality of the medium causing

[3] Authors assumed a complementary step of photo-fermentation for further oxidation of by-products of dark fermentation.

[4] Environmental impact for pre-treatment of a biomass will vary depending on the nature of biomass and the method of pre-treatment used. Hence, the pre-treatment phase has been omitted from the discussion.

undesirable effects on microbial biomass and H_2 yields (Ljunggren et al., 2011a). In a more recent study, use of beverage wastewater and agricultural waste for biological hydrogen production was forecasted to be commercially feasible with an annual 'return on investment' rate of 60% and 39% respectively (Li et al., 2012).

2.6.3. Other aspects

Given that H_2 needs a unique and costly distribution infrastructure; a decentralized model of production can be imagined for a biomass-dependent thermophilic H_2 process. A decentralized production will also benefit from a locally available market. Alternatively, thermophilic biohydrogen can be produced in an add-on plant to another industrial process. For example, by-products and waste heat of a sugar factory can be used for the production of biohydrogen (Markowski et al., 2010).

3. Physiological intricacies of *C. saccharolyticus*

Caldicellulosiruptor saccharolyticus was isolated from a thermal spring of Taupo area of the Taupo-Rotorua thermal region of New Zealand and was originally classified as *Caldocellum saccharolyticum* (Sissons et al., 1987). However, it was later re-classified as *C. saccharolyticus* and was attributed as an asporogenous, extremely thermophilic, strictly anaerobic and Gram-positive bacterium capable of growing at any temperature ranging from 45-80 (T_{opt} = 70 °C) and pH ranging from 5.5-8.0 (pH_{opt} = 7) (Rainey et al., 1994). Ever since, a number of species have been added to the genus *Caldicellulosiruptor* (Bredholt et al., 1999; Hamilton-Brehm et al., 2010; Huang et al., 1998; Miroshnichenko et al., 2008; Onyenwoke et al., 2006; Yang et al., 2010). However, *C. saccharolyticus* still remains most studied thermophilic biohydrogen producer due to variety of beneficial features it possesses (Bielen et al., 2013; Pawar & van Niel, 2013).

3.1. Distinct metabolic features of *C. saccharolyticus*

3.1.1. Hydrolytic capacity and growth on renewable feedstocks

As discussed above, the natural habitat of *C. saccharolyticus* does not have monomeric sugars but complex (hemi)cellulosic polymers. As a result, *C. saccharolyticus* has evolved with an extra-ordinary ability to hydrolyse a variety of naturally occurring polymers, such as lignocellulosic materials (Blumer-Schuette et al., 2010). Interestingly, within the genus *Caldicellulosiruptor*, *C. kronotskyensis* followed closely by *C. saccharolyticus*, has the best inventory of various different *glycosyl hydrolases* (Blumer-Schuette et al., 2010). Nonetheless, *C. saccharolyticus* can hydrolyse various α- and β- linked di-, oligo-, and poly-saccharides, such as starch, maltose, sucrose, pullulan, pectin, trehalose, xylan and (hemi)celluloses (Rainey et al., 1994). So far, *C. saccharolyticus* has been reported to grow and produce H_2 on hydrolysates of wheat

straw (**Paper II**), Miscanthus (de Vrije et al., 2009), paper sludge (Kadar et al., 2003), barley straw and corn stalk (Panagiotopoulos et al., 2009), juices of sweet sorghum (Ivanova et al., 2009) and sugar beet (Panagiotopoulos et al., 2010), as well as untreated wheat straw, sugarcane bagasse, leaves of maize or *Silphium trifoliatum* (Ivanova et al., 2009), and potato steam peels (Mars et al., 2010).

3.1.2. Absence of carbon catabolite repression (CCR)

The products of hydrolysed complex carbohydrates i.e. the soluble mono-, di- or oligo-saccharides enter *C. saccharolyticus* via ABC transporters at the expense of ATP. *C. saccharolyticus* also possesses a fructose-specific PTS transporter (van de Werken et al., 2008). Out of 177 annotated ABC transporters, only 24 have been assigned with substrate specification, meaning that most ABC transporters have a broad substrate range and also some substrates can be transported by multiple transporters (VanFossen et al., 2009). When *C. saccharolyticus* was grown on a mixture of sugars including pentoses and hexoses, it displayed the ability of co-utilization of all these sugars confirming the absence of CCR ((van de Werken et al., 2008; VanFossen et al., 2009) and **Paper II**). The co-utilization of sugars was also observed with the hydrolysates of renewable feedstocks containing a mixture of sugars ((de Vrije et al., 2009; de Vrije et al., 2010) and **Paper II**). Although *C. saccharolyticus* can co-utilize various sugars, it has preference for some sugars over others in following order fructose > arabinose > xylose > mannose > glucose > galactose (VanFossen et al., 2009). As discussed in Section 2.4, the absence of CCR is very important property in making *C. saccharolyticus* very desirable as industrial biohydrogen producer.

3.1.3. PPi as a central energy carrier

Inorganic pyrophosphate (PPi) is formed as a by-product during DNA replication and during RNA, protein and lipid synthesis (Heinonen, 2001). These reactions generally occur close to thermodynamic equilibrium. Thus, subsequent hydrolysis of PPi possessing a high negative Gibbs free energy is required to enable continuation of biosynthesis of the afore-mentioned essential macromolecules (Heinonen, 2001). Generally, a cytosolic pyrophosphatase performs the hydrolysis of PPi in most organisms, which is lacking in *C. saccharolyticus* (Bielen et al., 2010) as in many other thermophiles. Instead *C. saccharolyticus* possesses a membrane bound H+ translocating pyrophosphatase, which helps preserve the energy of PPi hydrolysis in the form of a proton motive force. Moreover, PPi can also be used as an energy

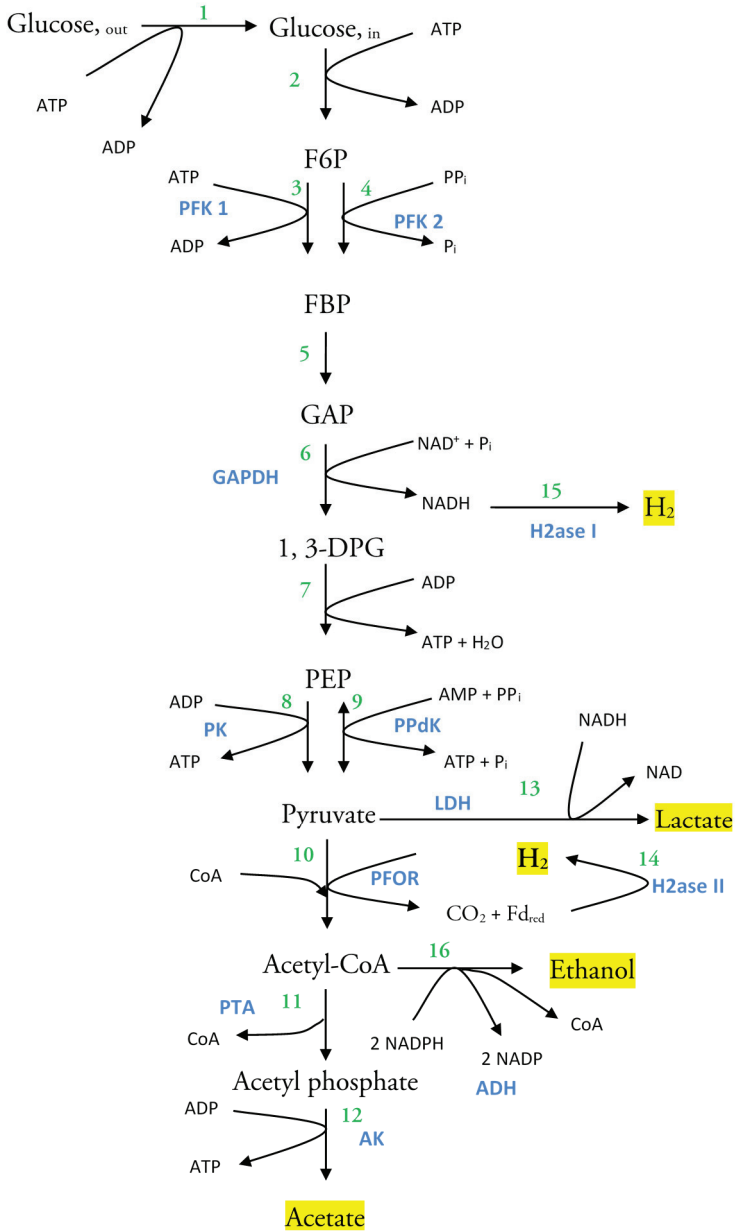

Figure 2 Simplified schematic of central carbon metabolism in *C. saccharolyticus.* PFK, phosphofructokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H2ase I, Fe-only hydrogenase; PK, pyruvate kinase; PPdK, pyrophosphate dependent kinase; LDH, lacate dehydrogenase; PFOR, pyruvate:ferredoxin oxidoreductase; H2ase II, NiFe hydrogenase; PTA, phosphoacetyltransferase; ADH, alcohol dehydrogenase; AK, acetate kinase.

carrier in central carbon metabolism of *C. saccharolyticus* for the synthesis of - i) FBP catalysed by PPi –dependent PFK and ii) pyruvate catalysed by PPDK (Reactions 4 and 9 respectively, Figure 2) (Bielen et al., 2010). Thus, as PPi is generally produced during anabolism and ATP during catabolism, Willquist and co-workers argued that the use of PPi as an alternative energy carrier to ATP, provides metabolic flexibility to *C. saccharolyticus* (Willquist et al., 2010). Interestingly, during assimilation of sulphate by *C. saccharolyticus*, 2 moles of ATP are used in two different reactions, one of which is catalysed by sulphate adenylyltransferase producing 1 mole of PPi as a by-product, and thus reducing net energy spent during the process (**Paper III**). Furthermore, PPi also takes part in regulating metabolic flux distribution at the pyruvate node by controlling the activity of the enzyme lactate dehydrogenase (Willquist & van Niel, 2010).

3.1.4. Ability to withstand high P_{H2}

Thermodynamics and P_{H2}

The genome of *C. saccharolyticus* possesses genes coding for two distinct *hydrogenases* – a NADH-dependent Fe-only hydrogenase (Csac 1860-1864), and a Fd_{red}-dependent NiFe hydrogenase (Csac 1535-1539) and several genes for maturation of the hydrogenase complex (Csac 1540-1545) (van de Werken et al., 2008). As discussed earlier (Section 2.3.1), from a strict thermodynamic perspective, the possession of an Fd-dependent hydrogenase enables *C. saccharolyticus* to tolerate high P_{H2} (**Paper I**). However, due to a variety of reasons, as argued by Willquist and co-workers, thermodynamic constraint alone cannot be a criterion to determine the critical P_{H2} affecting H_2 producers (Willquist et al., 2010). Nevertheless, during the study described in **Paper III**, it was also discovered that the choice of the sulphur source in the growth medium does not affect the ability of *C. saccharolyticus* to tolerate high P_{H2}.

P_{H2} and concentration of H_2 in aqueous phase ($H_{2,aq}$)

Generally, due to practical difficulties in measuring the concentration of H_2 in the aqueous phase ($H_{2,aq}$), the P_{H2} in the gaseous phase of the reactor, instead is considered as a factor affecting the behaviour of a microorganism when reactors are operating without employing any means of H_2 removal (Pauss et al., 1990). However, due to liquid-to-gas mass transfer limitations of H_2, it can easily supersaturate in the aqueous phase. Therefore, the P_{H2} of the gas phase does not correlate with the corresponding $H_{2,aq}$ (Pauss et al., 1990). A previous study revealed that inhibition of H_2 production is mostly caused by $H_{2,aq}$ rather than the P_{H2}, and

the former is dependent on the liquid-to-gas mass transfer rate of H_2 (Ljunggren et al., 2011b). In the CSTR, the mass transfer rate, i.e. removal of H_2 from the aqueous phase, can be improved by two means - by sparging the reactor with an inert gas and/or by stirring the reactor. When a combination of sparging and stirring is applied in the continuous cultures of *C. saccharolyticus* in a CSTR, H_2 yields close to the theoretical maximum were obtained (**Paper III**, Figure 3). However, consistent with a previous study (Ljunggren et al., 2011b), inhibition to H_2 production was more dependent on 'sparging' than 'stirring' (**Paper I and IV**, Figure 3).

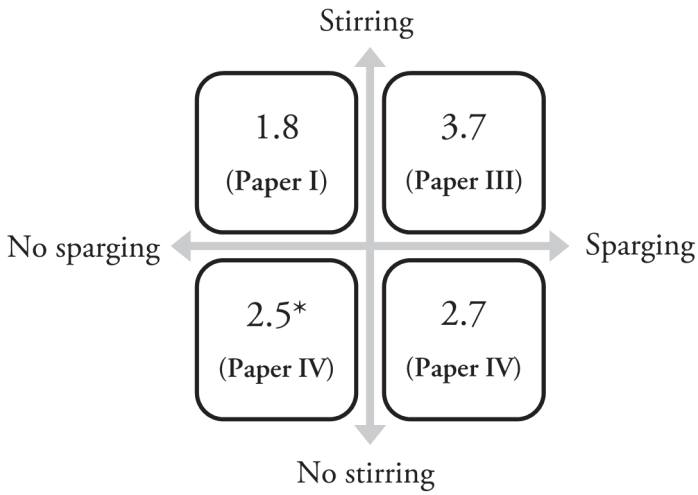

Figure 3 H_2 yield in continuous cultures performed in a CSTR (Note: the cultivation medium used for each condition was slightly different in its composition). *The experiment was performed with a co-culture of *C. saccharolyticus* and *C. owensensis* in UA reactor containing granular sludge (for more explanation see Section 4.3.2)

3.1.5. Ability to grow in a minimal medium

In a previous study it was experimentally proven that *C. saccharolyticus* is prototrophic to all the amino acids required in protein synthesis or other cellular functions and thus, does not need addition of complex substrates, such as yeast extract and/or peptone in its growth medium (Willquist & van Niel, 2012). As discussed earlier (Section 2.5.2), this allowed getting rid of the most expensive component, i.e. yeast extract, from the cultivation medium of *C. saccharolyticus*. Moreover, it also allowed experiments in a chemically well-defined growth medium for more precise understanding of the physiological response of *C. saccharolyticus* to a particular condition (**Paper III and V**). Furthermore, the ability to grow a

chemically well-defined growth medium enabled development of uracil-auxotrophic strains of *C. saccharolyticus* (**Paper VI**).

In addition to yeast extract, cysteine.HCl is also an expensive ingredient used traditionally in the cultivation medium of *C. saccharolyticus* as a– i) reducing agent (Sissons et al., 1987), and ii) sulphur source (Zeidan, 2011). The evaluation of assimilatory sulphur metabolism in *C. saccharolyticus* revealed that, not only cysteine.HCl can cause emissions of hazardous H_2S gas, but also is not essential for growth and H_2 production (**Paper III**). Moreover, relatively inexpensive inorganic sulphate salts could successfully replace cysteine.HCl as a primary sulphur source in the growth medium of *C. saccharolyticus* without any apparent emission of H_2S, thus improving safety and economic feasibility (**Paper III**).

3.2. Limitations of *C. saccharolyticus* as a H_2 producer

In addition to understanding the fundamental physiological properties in *C. saccharolyticus*, this study also dealt with some of the key developments needed to improve the organism's potential as an efficient H_2 producer.

3.2.1. Vulnerability to high osmotic culture medium

Any industrially applicable microorganism should be able to tolerate a medium with high substrate concentrations (*Property H*). However, *C. saccharolyticus* is highly vulnerable to increased substrate and product concentration in a culture medium. Indeed, growth of *C. saccharolyticus* is completely ceased in a medium containing total solute (salts and sugar) concentration above 400-425 mM (van Niel et al., 2003). Moreover, the medium with osmolality above approx. 220 mOsmol/KgH_2O induced early cell lysis in batch cultures of *C. saccharolyticus* (Willquist et al., 2009).

3.2.2. Low volumetric H_2 productivity

From a techno-economic perspective, volumetric H_2 productivity (Q_{H2}) is a significant criterion, the higher the Q_{H2}, the better the process economics. Despite considerable efforts, mainly pertaining to reactor design, Q_{H2} of the

cultures of *C. saccharolyticus* still remain at least an order of magnitude lower than the desired values (Bielen et al., 2013).

3.2.3. Unavailability of tools for genetic modification

Availability of tools for genetic manipulations is quintessential for any organism desired for industrial applications (*Property B*). However, genetic modification of thermophilic microorganisms, including *C. saccharolyticus*, has been a challenge to researchers worldwide (Noll & Vargas, 1997).

4. Measures to improve volumetric H_2 productivity

4.1 The yield vs. productivity conundrum

As described above (Section 2.1) the thermophilic H_2 producers, such as *C. saccharolyticus*, are better equipped to produce H_2 with yield approaching theoretical maximum. The values of H_2 yield (Y_{H2}), i.e. the amount of H_2 obtained per unit of substrate consumed, signify the conversion efficiency of a process. Whereas, the volumetric H_2 productivity (Q_{H2}) signifies the amount of H_2 produced per unit reactor volume per unit time. Unfortunately, the Y_{H2} and Q_{H2} have a reciprocal relationship, meaning that any attempt in improving either one of them may result in diminution of other (Rittmann & Herwig, 2012).

For expensive feedstocks, such as starch (originating from 'food'), the lower Y_{H2} would mean higher production cost, which is undesirable. However, as *C. saccharolyticus* can utilize relatively cheap feedstocks, such as renewable lignocellulosic materials, waste streams from food industry or municipal waste (Section 3.1.1), improvement in the Q_{H2} may be more important from an industrial perspective. Besides, thermophilic biohydrogen production can be accompanied by a complementary process to convert remaining sugars and by-products (Section 2.5 and **Paper II**). Therefore, a substantial part of this study was devoted to identify and implement the different measures to improve the Q_{H2}.

4.2 Factors affecting H_2 productivity

The Q_{H2} can be enhanced considerably by improving the substrate conversion rate (SCR). The SCR in turn is dependent on the substrate loading rate (SLR) and the cell density of the culture (figure 4).

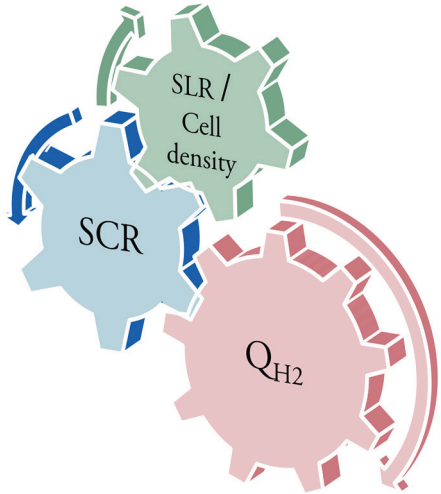

Figure 4 Measures to improve volumetric H_2 productivity

The SLR can be increased by two means –i) by decreasing the hydraulic retention time (HRT) at relatively low substrate concentration (**Paper IV**) or ii) by increasing the substrate concentration in the medium at relatively high HRT (**Paper V**). On the other hand, the cell density in the culture can be improved by –i) employing reactor systems capable of retaining the cells (**Paper IV**), and/or ii) by employing methods of cell retention, e.g. promoting biofilm formation by the cells (**Paper IV**).

4.3 Approach I: Low HRT and Biofilm formation

4.3.1 Biomass retention and reactor type

Most of the research efforts pertaining to thermophilic H_2 producers is directed towards understanding their physiological attributes (Kengen et al. 2009). Invariably, such studies need to be performed in well-controlled laboratory conditions, such as the use of a N_2-sparged, continuously stirred tank reactor (CSTR). However, as the CSTR does not allow cell retention, it is limited in its ability to allow for low HRTs and higher SCR. These limitations have a direct consequence in restricting the Q_{H2} to considerably lower values (Pawar & van Niel, 2013). On the other hand, these limitations of the CSTR have promoted significant

investigations into finding advanced bioreactor systems that allow biomass retention and consequently low HRT (Table 5; for a more extensive list of reactor studies see (Ren et al. 2011)). In case of *C. saccharolyticus*, application of trickle-bed reactor reported the highest ever Q_{H2} (i.e. 22 mmol/L/h) obtained for its continuous cultures (van Groenestijn et al., 2009). During the study described in **Paper IV**, a maximum Q_{H2} of approx. 20 mmol/L/h with corresponding Y_{H2} of 2.5 mole H_2/mole of glucose consumed was obtained when *C. saccharolyticus* was grown in a co-culture with *Caldicellulosiruptor owensensis* in an UA reactor containing granular sludge.

4.3.2 Sparging and Q_{H2}

For most of the studies with *C. saccharolyticus*, N_2 has been a popular choice as a sparging gas for stripping the culture medium off $H_{2, aq}$ (Bielen et al., 2013; Willquist et al., 2010). Although N_2 is inexpensive and abundant, its inert nature makes it very difficult to be separated from H_2 and thus unsuitable at an industrial scale (van Groenestijn et al., 2002). As discussed earlier (Section 3.1.4), $H_{2, aq}$ influences the growth and H_2 production in *C. saccharolyticus*. Therefore, various different H_2 removal strategies were studied to evaluate their effect on Q_{H2} (Table 6).

Table 6 The Q_{H2} (mmol/L/h) of *C. saccharolyticus* under various sparging conditions.

Sparging gas / condition	Flow rate ($L \cdot h^{-1}$)	Q_{H2} @ $D = 0.05\ h^{-1}$	Reference
N_2	6	5.2	Paper I
67%CH_4 (N_2)+33%CO_2*	6	2.1	Paper II
CH_4(N_2)*	6	5.2	Paper II
No sparging, CSTR	-	2.4	Paper I
No sparging, UA**	-	4.6	Paper IV

*This study revealed that CH_4 is as inert as N_2 to the growth of and H_2 production by *C. saccharolyticus*, ** the experiment was performed with a co-culture of *C. saccharolyticus* and *C. owensensis* in UA reactor containing granular sludge.

When a gas mixture mimicking the flue gas (CH_4 + CO_2) of methanogenic anaerobic digester was used for sparging, the Q_{H2} was reduced by more than 50% of the Q_{H2} obtained with N_2 or CH_4 sparging (**Paper II**, Table 6). This reduction in Q_{H2} was mainly due to the detrimental effect of CO_2 saturation on *C. saccharolyticus* in its culture medium (Willquist et al., 2009). Moreover, the use of 'CH_4 + CO_2' (**Paper II**) had a similar effect on the Q_{H2} when no sparging was applied in a CSTR (**Paper I**). Interestingly, the Q_{H2} obtained in the UA reactor in absence of sparging was almost twice as that of the CSTR in similar conditions (Table 4). The reason being that the UA reactor allows re-circulation of culture medium which aids in improving the substrate accessibility to the cells as well as it can increase the turbulence in the reactor facilitating the removal of $H_{2, aq}$ (**Paper IV**).

4.3.3 Designed co-cultures and biofilm formation

The application of designed co-cultures of H_2 producers that display synergies and enhance the metabolic features, thereby increasing the H_2 yield and substrate conversion efficiencies is emerging as an interesting alternative to pure and undefined cultures (Pawar & van Niel, 2013). So far, only a handful of researchers have attempted to evaluate the potential of such designed co-cultures. *Clostridium thermocellum* is very efficient in hydrolysing cellulose. However, it is incapable of a complete utilization of the products of hydrolysis i.e. glucose and cellobiose. Moreover, building up of glucose and cellobiose inhibits the cellullases in its culture (Johnson et al., 1982). On the other hand, *Thermoanaerobacterium thermosaccharolyticum* is unable to hydrolyse cellulose but is very efficient in utilization of glucose and cellobiose (Baskaran et al., 1995). This explains the finding of a natural co-culture of *T. thermosaccharolyticum* and *C. thermocellum* from decomposing wheat straw (Liu et al., 2008). Indeed, a synthetic co-culture of *C. thermocellum* and *T. thermosaccharlyticum* performed in batch mode with cellulose as a main substrate, obtained higher H_2 yields (1.8 mol H_2/mol glucose) than *C. thermocellum* alone (0.8 mol H_2/mol glucose) mainly due to higher conversion efficiency (Liu et al., 2008). Similarly, when grown in synthetic co-cultures, *Caldicellulosiruptor* species displayed synergistic behaviour despite each originating from a different habitat ((Zeidan et al., 2010) and **Paper IV**). Indeed, the co-cultures of *C. saccharolyticus* and *C. kristjanssonii* exhibited an exceptional stability in continuous culture operated at different dilution rates even when sharing a single substrate (Zeidan et al., 2010). Similarly, when *C. saccharolyticus* and *C. owensensis* were grown in the presence of each other's supernatant, their growth and H_2 production were markedly improved (**Paper IV**).

It is generally claimed that biofilms can improve the reaction rates in various industrial bioconversion processes (Qureshi et al., 2005). Furthermore, van Groenestijn and co-workers argued that the biofilms will be an effective means of improving cell density and consequently the Q_{H2} (van Groenestijn et al., 2002). Evidently, when *C. saccharolyticus* and *C. owensensis* were grown in a co-culture performed in an UA reactor with granular sludge, their biofilms improved the Q_{H2} by at least 2-fold than that of their pure cultures, which failed to form substantial amounts of biofilms (**Paper IV**). In addition, elevated intracellular levels of a secondary messenger, c-di-GMP were found to encourage biofilm formation by the *Caldicellulosiruptor* species (**Paper IV**).

4.4 Approach II: Osmo-adaptation of *C. saccharolyticus*

The natural habitats of the majority of thermophilic H_2 producers is low in simple mono- or di-meric sugars but abundant in complex polysaccharides. Thus, these microorganisms usually possess a variety of hydrolases to breakdown these polysaccharides (Blumer-Schuette et al., 2008). The released mono- and disaccharides are consumed rapidly, thus the organisms are rarely exposed to environments with high sugar concentrations (Willquist et al., 2010). Consequently, growth of these microorganisms is severely influenced by the osmotic pressure exerted by high sugar concentrations (Ljunggren et al., 2011b).

4.4.1 Development of the osmotolerant strains of *C. saccharolyticus*

A previous study revealed that the Q_{H2} of *C. saccharolyticus* declined when the concentration of sucrose in the medium was increased by more than 30 mM (van Niel et al., 2003). Moreover, the growth rates of *C. saccharolyticus* were reduced by at least 60% when the medium osmolality increased to more than 220 mOsmol/KgH_2O (Willquist et al., 2009). Therefore, to enable *C. saccharolyticus* to grow in a medium containing high amounts of substrate and/or by-products, it was adapted to increased medium osmolality (>600 mOsmol/KgH_2O) by using a method of evolutionary engineering (Fig. 5, **Paper V**).

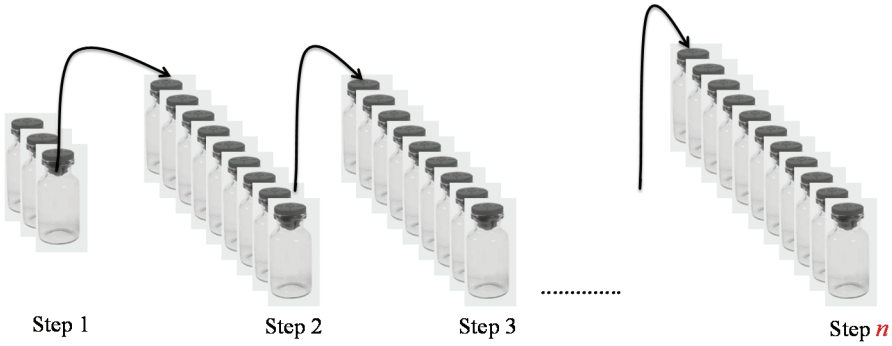

Figure 5 Step-wise adaptation of *C. saccharolyticus* to increasing osmotic pressure

Thus, two osmotolerant strains, viz. *C. saccharolyticus* G10 and *C. saccharolyticus* AG6, were obtained (**Paper V**). The *C. saccharolyticus* G10 was adapted to a medium containing up to 100 g/L of glucose and the *C. saccharolyticus* AG6 was adapted to a medium containing approx. 13.2 g/L of sodium acetate and 30 g/L of glucose.

4.4.2 Evaluation of the osmotolerant strains

When the adapted strains were grown in continuous cultures performed in CSTR, they displayed a remarkable stability in high-osmotic medium. However, when they were grown in a low-osmotic medium, both the adapted strains displayed decreased fitness compared to the wild-type strain (**Paper V**). Nevertheless, one of the ancestors of the strain *C. saccharolyticus* G10, *C. saccharolyticus* G5 was able to attain approx. 9 mmol/L/h of SCR which resulted in Q_{H2} values of approx. 20.7 mmol/L/h (**Paper V**), the highest ever reported for continuous cultures performed in a CSTR using *C. saccharolyticus* (Bielen et al., 2013).

Among all the *Caldicellulosiruptor* species, *C. saccharolyticus* contains the most number of insertion sequence (IS) elements (Chung et al., 2013b), which function as mobile genetic elements (MGEs). MGEs are known to play an important part in adaptation of bacteria to stressful environments (Blot, 1994; Top & Springael, 2003). Indeed, genome-wide transcription analysis of adapted strains suggests that the IS elements may have produced random but beneficial mutations in the genomes conferring osmotolerance (**Paper V**).

4.5 Concluding remarks

Formation of biofilm is beneficial in enhancing the substrate conversion rate as well as the H_2 productivity regardless of the reactor type used (**Paper IV**). The UA reactor offers a suitable alternative to the CSTR for cultivation of H_2 producers and also produce H_2 at higher rates (**Paper IV**). An osmotolerant strain *C. saccharolyticus* G5, produced H_2 at higher rates in an optimized medium (**Paper V**). However, more research is needed especially pertaining to medium composition and optimization to attain complete substrate conversion, which may further improve H_2 productivity by several folds. On the other hand, the means employed to increase biomass concentration suffered a set-back due to undesirable lactic acid production (**Paper IV**). Further developments at genetic level may help reduce the lactate production. Ideally, a combination of both approaches, i.e. promoting biofilm formation of osmotolerant strains of *Caldicellulosiruptor* species, holds a huge potential in increasing H_2 productivities.

5. Genetic modification of *C. saccharolyticus*

The research and development pertaining to physiology of the popular microbes, such as *Saccharomyces cerevisiae* and *Escherichia coli* has improved by leaps and bounds in past few decades creating numerous industrial applications. Arguably, the development of various kinds of genetic tools for these microbes has played a major role in their individual development as an industrial workhorse. Perhaps similarly, development of genetic tools for *C. saccharolyticus* may facilitate its development as an efficient H_2 producer. Indeed, tools for genetic modification are vital to obtain required knowledge in the most convenient way. However, only few tools have been successfully obtained for the genetic modification of the known thermophilic H_2 producers (Table 7, for a complete list see (Raj et al., 2012)). Moreover, these tools are mostly organism specific, thus limiting their application in other H_2 producers. Researchers face a number of challenges in developing the tools for genetic modification in thermophilic H_2 producers.

5.1 Challenges in genetic modification of thermophiles

Apart from the technical difficulties in growing strictly anaerobic thermophiles on a solid media, thermophiles in general pose very specific challenges in developing genetic tools, as listed below.

5.1.1 Availability of selection marker

Historically, antibiotic resistance markers and auxotrophic markers have been the most popular choice as a marker for selecting the cells displaying relevant phenotype after a successful genetic transformation of an organism (Noll & Vargas, 1997).

Application of antibiotic selection markers in thermophiles is limited due to – i) instability of the antibiotics and ii) unavailability of proteins conferring resistance to these antibiotics, under the conditions appropriate for their cultivation (Allers & Mevarech, 2005; Noll & Vargas, 1997; Tyurin et al., 2006). On the other hand, auxotrophic markers are mostly applicable in organisms capable of growing in chemically defined growth medium (Noll & Vargas, 1997). Despite of these challenges, suitable markers have been developed for a few thermophilic microorganisms (Allers & Mevarech, 2005; Raj et al., 2012).

5.1.2 Availability of native thermophilic plasmids

In general, a site of 'origin of replication' (termed as *ori*), a protein (i.e. Rep) needed in the initiation of replication, and genes related to replication play a significant role in replication of plasmid inside a host (del Solar et al., 1998). In addition to these elements, plasmids also depend upon the replication machinery of the host (del Solar et al., 1998). Thus, to develop a replicating vector for genetic modification of any organism, it is important to find native plasmids harboured by the organism(s) closely related to the desired host. A very few thermophilic Gram-positive bacteria are known to harbour native plasmids (Belogurova et al., 1991; Blumer-Schuette et al., 2011; Clausen et al., 2004; Kurose et al., 1989; Tsoĭ et al., 1987; Weimer et al., 1984).

5.1.3 Restriction-modification system

In bacteria, a restriction-modification (R-M) system primarily functions as a defence system against the invading bacteriophages. It consists of a combination of enzymes with opposing activities i.e. a restriction endonuclease and its corresponding (cognate) DNA methyltransferase (Wilson & Murray, 1991). Based on the mode of action, symmetry of the recognition sequence, cofactor requirements, and the site of cleavage relative to its recognition site, the R-M systems were originally classified in to three different groups: type I, II and III (Yuan et al., 1982). Very recently, another group was added to separate methyl-dependent restriction enzymes from others, to be identified as type IV R-M system (Roberts et al., 2003). Similarly, based on the site of methylation, the methyltransferases have been classified into three different classes: C5-cytosine-specific (m5C), N4 cytosine-specific (m4C) and N6 adenine-specific (m6A) (Wilson & Murray, 1991). The native restriction endonucleases do not cut the organisms own genomic DNA. This is due to the presence of a cognate

Table 7 Examples of the tools developed for genetic modification in thermophilic H_2 producers

Plasmid	Description	Host	Gene of Interest	Gene source	References
pIKM3	ColEH1; ORF2;Amp^r; MLS^r	*T. saccharolyticum* JW/SL-YS485	*manA*	*manA*:*Caldicellulosiruptor* Rt8B4	(Mai et al., 2000)
pUDT2	Replicating; *pyrF*; double cross-over;	*T. kodakarensis* KOD1	*trpE*	*trpE:T. kodakarensis* KOD1	(Sato et al., 2003)
pDCW89	Replicating; *pyrBCF*	*C. hydrothermalis* Δ*pyrBCF*	*pyrBCF*	*pyrBCF:C. bescii*	(Chung et al., 2012)
pDCW121	pDCW88; Apr^r; Δ*ldh*	*C. bescii* Δ*pyrBCF*	*ldh (deletion)*	*pyrBCF:C. bescii*	(Cha et al., 2013)
pDH10	pRQ7; Amp^r; Kan^r	*T. neapolitana, T. maritima*	*kan*	NA*	(Han et al., 2012)
pYS3/4	pYS2; HMG CoA *reductase* (*hcr*)	*P. furiosus*	*hcr*	*hcr:P. furiosus*	(Waege et al., 2010)

*Purchased from Biotools, BM labs, Madrid, Spain

methyltransferase. The methyltransferases introduce a methyl group onto a particular nucleotide in DNA preventing the activity of the corresponding restriction enzyme (Old & Primrose, 1981). Thus, it is essential to understand the R-M system of the host to find a compatible methyltransferase protecting the exogenous DNA from host restriction endonucleases.

5.2 Genetic modification of *C. saccharolyticus*

Within the genus *Caldicellulosiruptor*, so far, only *C. bescii* (Cha et al., 2013; Chung et al., 2012) and *C. hydrothermalis* (Chung et al., 2013a) have been reported with successful genetic modification. However, vectors designed in these studies could not be applied to perform genetic modifications of any other species of genus *Caldicellulosiruptor* including *C. saccharolyticus* (Chung et al., 2013a), mostly due to differences in their individual R-M systems (**Paper VI**).

Among all the *Caldicellulosiruptor* species, only *C. kristjanssonii* and *C. bescii* have been reported to harbour native plasmids (Table 8). However, only the plasmid pCALKR01 could survive in the presence of the restriction endonucleases of *C. saccharolyticus*, due to methylation by its own m5C methyltransferases (Calkr_2643, **Paper VI**).

Table 8 The native plasmids harboured by the members of genus *Caldicellulosiruptor*

Plasmid	Size	Host organism	Reference
pCALKR01	15.7	*C. kristjanssonii*	(Blumer-Schuette et al., 2011)
pBAL	8.2	*C. bescii*	(Clausen et al., 2004)
pBAS2	3.6	*C. bescii*	(Clausen et al., 2004)

The process of evolutionary engineering was also applied to develop several stable uracil-auxotrophic strains of *C. saccharolyticus* (*C. saccharolyticus* URA$^-$, **Paper VI**), any of which can be used as a host to perform genetic modifications by using uracil-prototrophy for counter-selection. However, a complete genotypic characterization of these *C. saccharolyticus* URA$^-$ needs to be performed to identify and locate the deletions.

In conclusion, the knowledge and tools developed during this study (**Paper VI**) can help in designing various combinations of vectors based on pCALKR01, pBAL or pBAS2, which can be methylated with m5C methyltransfearses to overcome the R-M system of *C. saccharolyticus*.

6. An ideal hydrogen producer

The investigations into the physiological and metabolic features of most thermophilic H_2 producers and also the implications of these features in enabling them as efficient H_2 producers will continue to be explored. To aid in screening new microorganisms and/or developing the known organisms in search of an ideal H_2 producing microorganism, the following combination of *properties* have been proposed recently (Pawar & van Niel, 2013), that such an ideal H_2 producer may possess – "A) thermophilic (Section 2.1), B) has specific vectors/tools designed for genetic modification(s) (Section 3.2.3), C) possesses Fd-dependent *hydrogenases*

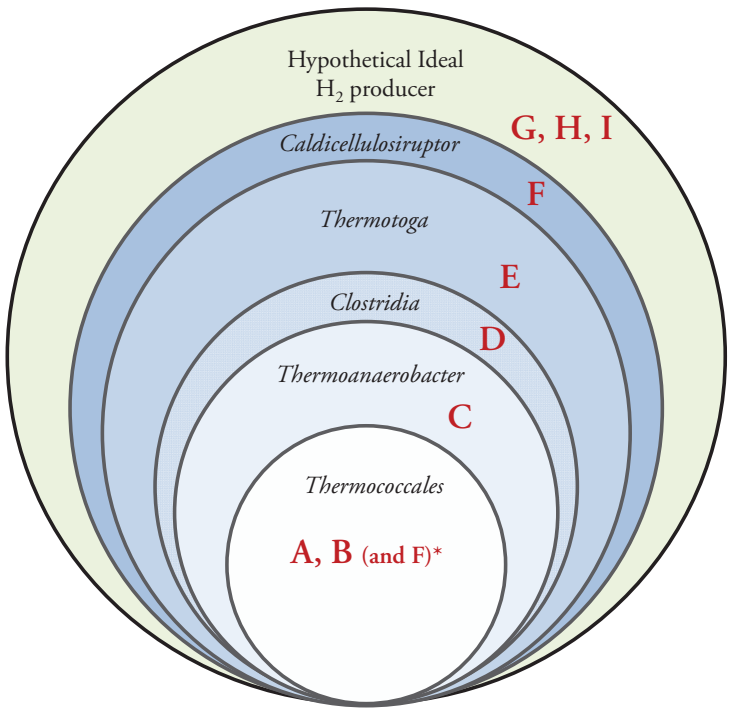

Figure 6 A Venn diagram displaying a comparison between distinguished H_2 producers with respect to desirable properties (A to I) an ideal H_2 producer may possess (Adopted from (Pawar & van Niel, 2013)). *, Note: the property F is also present in *Thermococcales* and is indeed absent from most members of other genera

(Section 2.3.1), D) is not auxotrophic to any amino acids (Section 2.4), E) has ability to degrade a wide range of biomass (Section 2.4), F) can metabolize multiple sugars simultaneously (absence of carbon catabolite repression) (Section 2.4), G) when under stress shifts metabolism to useful by-products (Section 2.2), H) is tolerant to high osmotic stress exerted by high substrate/by-product concentrations (Section 3.2.1) and, I) is tolerant to presence of oxygen (Section 2.3.1)". Evidently, among the well-known H_2 producers, organisms belonging to the genera *Caldicellulosiruptor* and *Thermotoga* are next to being the ideal H_2 producers (Table 4 and Fig. 6).

As described earlier, the wild-type *C. saccharolyticus* possesses the properties A, C, D, E and F (**Chapter 3, Paper I, II and III**). Moreover, *C. saccharolyticus* can sustain growth without the need of any means of removal of H_2 from the reactor (**Paper I**) and can promote and participate in biofilm formation under appropriate conditions (**Paper IV**). In addition to this, *C. saccharolyticus* G10 and *C. saccharolyticus* AG6 have displayed tolerance to high osmotic stress i.e. property H (**Chapter 4** and **Paper V**). Furthermore, the *C. saccharolyticus* URA^- can make a potential host for genetic modification by using a vector based on the knowledge and tools obtained in this study (**Chapter 5** and **Paper VI**) i.e. property B. Thus, if all the properties of the three strains i.e. *C. saccharolyticus, C. saccharolyticus* G10 and *C. saccharolyticus* URA^- can be brought together, the resulting strain may be as closest as it can get to an ideal H_2 producer.

7. Conclusions

The main conclusions of this thesis are -

- *C. saccharolyticus* can sustain its growth in the absence of any means of removal of H_2 from the reactor, albeit displaying lower H_2 yields caused by a metabolic shift to lactate and/or ethanol production (**Paper I**, **III**, and **IV**).
- *C. saccharolyticus* can utilize sugars in wheat straw hydrolysate effectively for its growth and H_2 production. The residual sugars and acids produced can subsequently be converted to methane by anaerobic digestion, thereby proving its ability to complement thermophilic dark fermentation (**Paper II**).
- Methane can replace N_2 as a sparging gas for removal of H_2 from the culture, without affecting the growth and H_2 production by *C. saccharolyticus* (**Paper II**).
- Relatively inexpensive inorganic sulphate salts can successfully replace cysteine·HCl as a primary sulphur source in the growth medium of *C. saccharolyticus* (**Paper III**).
- Addition of a chelating agent such as, Nitrilotriacetic acid is essential to avoid precipitations in a chemically defined growth medium of *C. saccharolyticus* (**Paper III**).
- Biofilm formation by *Caldicellulosiruptor* species in a UA reactor can be an effective means to retain biomass thereby allowing higher volumetric H_2 productivities. However, *C. saccharolyticus* was able to form biofilm only when it was grown in a co-culture with *C. owensensis* under appropriate conditions (**Paper IV**).
- Evolutionary engineering is an effective means of developing stable mutant strains of *C. saccharolyticus*, viz. *C. saccharolyticus* G10, *C. saccharolyticus* AG6, and *C. saccharolyticus* URA^- (**Paper V** and **VI**), which is probably aided by the mobile genetic elements present in *C. saccharolyticus* (**Paper V**).

- One of the osmotolerant strains - *C. saccharolyticus* G5, was able to produce H_2 at the rate of 20.7 mmol/L/h, the highest ever reported for *C. saccharolyticus* grown in a CSTR containing a chemically optimized medium (**Paper V**).
- Methylation by C5-cytosine-specific methyltransferase can overcome the restriction-modification system of *C. saccharolyticus* in the development of a vector for genetic modification (**Paper VI**).
- Finally, the knowledge and newfound properties obtained in this study should be combined to create a strain of *C. saccharolyticus* that will fulfil nearly all the requirements to make it into an ideal H_2 producer.

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Paper I

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Reassessment of hydrogen tolerance in *Caldicellulosiruptor saccharolyticus*

Karin Willquist, Sudhanshu S Pawar* and Ed WJ Van Niel

Abstract

Background: *Caldicellulosiruptor saccharolyticus* has the ability to produce hydrogen (H_2) at high yields from a wide spectrum of carbon sources, and has therefore gained industrial interest. For a cost-effective biohydrogen process, the ability of an organism to tolerate high partial pressures of H_2 (P_{H2}) is a critical aspect to eliminate the need for continuous stripping of the produced H_2 from the bioreactor.

Results: Herein, we demonstrate that, under given conditions, growth and H_2 production in *C. saccharolyticus* can be sustained at P_{H2} up to 67 kPa in a chemostat. At this P_{H2}, 38% and 16% of the pyruvate flux was redirected to lactate and ethanol, respectively, to maintain a relatively low cytosolic NADH/NAD ratio (0.12 mol/mol). To investigate the effect of the redox ratio on the glycolytic flux, a kinetic model describing the activity of the key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was developed. Indeed, at NADH/NAD ratios of 0.12 mol/mol (*Ki* of NADH = 0.03 ± 0.01 mM) GAPDH activity was inhibited by only 50% allowing still a high glycolytic flux (3.2 ± 0.4 mM/h). Even at high NADH/NAD ratios up to 1 mol/mol the enzyme was not completely inhibited. During batch cultivations, hydrogen tolerance of *C. saccharolyticus* was dependent on the growth phase of the organism as well as the carbon and energy source used. The obtained results were analyzed, based on thermodynamic and enzyme kinetic considerations, to gain insight in the mechanism underlying the unique ability of *C. saccharolyticus* to grow and produce H_2 under relatively high P_{H2}.

Conclusion: *C. saccharolyticus* is able to grow and produce hydrogen at high P_{H2}, hence eliminating the need of gas sparging in its cultures. Under this condition, it has a unique ability to fine tune its metabolism by maintaining the glycolytic flux through regulating GAPDH activity and redistribution of pyruvate flux. Concerning the later, xylose-rich feedstock should be preferred over the sucrose-rich one for better H_2 yield.

Keywords: *Caldicellulosiruptor saccharolyticus*, biohydrogen production, hydrogen tolerance, enzyme levels, glyceraldehyde-3-phosphate dehydrogenase kinetics, redox ratio

Background

In the continuous quest for an economically competitive biohydrogen production plant, it is important to obtain as high H_2 yields as possible [1]. The H_2 yields reported in literature for dark fermentation with various mesophilic microorganisms are usually in the range of 1-2 moles per mole of hexose [2-5], whereas a maximum empirical yield can be gained of 4 mol H_2/mol hexose [6]. One successful strategy to maximize H_2 yields is to carry out the fermentation with (hyper)thermophiles at elevated temperatures. This makes the H_2-generation reactions more energetically favourable [7] and has, therefore, a positive impact on H_2 yields [5,8]. Indeed, the highest H_2 yields reported to date, approaching the theoretical maximum, were obtained with (hyper)thermophiles [9-11].

Since H_2 is known to have an inhibitory effect on growth and its own production in a variety of microorganisms, including (hyper)thermophiles [5,8], maximizing fermentative H_2 yield is made possible by keeping the P_{H2} in the fermentation vessel sufficiently low. Normally, this is ensured by continuous stripping of the H_2 from the production broth using an inert gas, such as N_2 or He [12]. However, using an inert gas requires a subsequent energy-demanding gas-upgrading step [13]. Instead, CO_2 might offer an economic alternative [14]

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as it is a by-product of the fermentation process and can be more readily separated from H_2 [13]. However, stripping with CO_2 increases the osmolality of the fermentation broth, ultimately reducing the growth of the H_2-producing organism[15]. To avoid stripping, high H_2 yields have simply to be obtained at high P_{H2}.

Caldicellulosiruptor saccharolyticus is a strict anaerobic, extreme thermophilic bacterium that is able to produce nearly stoichiometric amounts of H_2 from glucose [9] and sucrose [16]. In addition, *C. saccharolyticus* has the unique ability to co-metabolize a wide spectrum of carbohydrates including both pentoses and hexoses [17,18], and to break down complex hemi-cellulosic materials as well as other complex polysaccharides [19-22]. The genome of this organism has been recently sequenced [17] facilitating improved discernments of its metabolic network.

High H_2 yields can only be achieved when acetate is the main metabolic by-product, since the formation of more reduced products, such as lactate and ethanol, drains electrons from H_2 production. In this work, we evaluated the influence of elevated P_{H2} on growth, the extent of lactate formation and accordingly H_2 yields, by *C. saccharolyticus* in batch as well as carbon-limited continuous cultures, with glucose as the main carbon and energy source. The effect of P_{H2} on *C. saccharolyticus* metabolism on pentoses (xylose) was also evaluated in batch cultures and was compared with previous results on disaccharides (sucrose; [23]). We demonstrate that, depending on the growth conditions, the organism can grow and produce H_2 at P_{H2} up to 67 kPa. The activity levels of three redox-related catabolic enzymes were compared in the presence and the absence of N_2 sparging and correlated with product distribution under each condition. In addition, a kinetic model was developed to examine the influence of the changes in the intracellular levels of NADH on the activity of a key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and is compared with other related organisms. The obtained results are analyzed, based on thermodynamic considerations, to understand the mechanism underlying the unique ability of *C. saccharolyticus* to grow and produce H_2 under relatively high P_{H2}.

Materials and methods

Microorganism and culture medium

C. saccharolyticus DSM 8903 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). A modified DSM 640 medium [15] was used for all cultivations throughout this work. Routine subcultures and inoculum development were conducted in 250-mL serum bottles containing 50-mL of medium. Anoxic solutions of different carbon sources were autoclaved separately and added to the sterile medium at the required concentration.

Fermentation setup

Cultures were grown in a jacketed, 3-L bioreactor equipped with an ADI 1025 Bio-Console and an ADI 1010 Bio-Controller (Applikon, Schiedam, The Netherlands) at a working volume of 1L, either in batch or continuous mode. The pH was maintained at 6.5 ± 0.1 at 70°C by automatic titration with 4 M NaOH. The temperature was thermostatically kept at 70 ± 1°C and the stirring rate was set to 250 rpm. A condenser with 5°C cooling water was fitted to the bioreactor's headplate. Prior to inoculation, the medium was sparged with N_2 and supplemented with an anoxic solution of cysteine-HCl at a final concentration of 1 g L^{-1} to render the medium completely anaerobic. For continuous cultivations, the bioreactor was started to be fed with fresh medium at the end of the logarithmic growth phase of the culture, having an identical composition to the batch start-up medium, except for cysteine-HCl (final concentration of 0.25 g/L in medium bottle) at the required dilution rate (*D*). Steady states were assessed after at least 5 volume changes based on the criteria of constant H_2 and CO_2 production rates and constant biomass concentration. Glucose was used as a primary substrate in all batch and continuous experiments at an initial concentration of 5 g/L, if not stated otherwise.

Three different experimental designs were applied: continuous flushing with 100 mL min^{-1} N_2 for continuous removal of produced H_2 (*Case I*); no gas sparging, with the bioreactor's gas outlet open leading to higher concentrations of H_2 in the headspace at 1 bar (*Case II*); no gas sparging, with the bioreactor's gas outlet closed allowing H_2 to accumulate and increasing the total pressure in the bioreactor (*Case III*). Gas samples from the headspace for H_2 and CO_2 determination and culture samples for monitoring growth, substrate consumption and product formation were regularly withdrawn during fermentation. At steady states, samples were taken for determining the NADH/NAD ratio and cell dry weight (CDW) and anaerobic culture samples for enzyme activity measurements as described previously [24]. Continuous cultivations were performed at the dilution rates of 0.05 h^{-1} and 0.15 h^{-1} in duplicate under both, '*Case I*' and '*Case II*', conditions.

Analytical methods

Headspace samples were analyzed for CO_2 and H_2 by gas chromatography, using a dual channel Micro-GC (CP-4900; Varian, Micro gas chromatography, Middelburg, The Netherlands), as previously described [11]. The results were analyzed with a Galaxie Chromatography Workstation (v.1.9.3.2). The optical density of the

culture was measured at 620 nm (OD_{620}) using a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). CDW was determined by filtration as previously described [24]. Glucose, acetate, lactate, succinate and ethanol were analyzed by HPLC (Waters, Milford, MA, USA) on an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA) at 45°C, with 5 mM H_2SO_4 (0.6 ml min^{-1}) as the mobile phase. The column was equipped with a refractive index detector (RID-6A; Shimadzu, Kyoto, Japan).

Preparation of cell extracts

Cell extracts (CE) were prepared anaerobically in duplicates using cells harvested from continuous cultures. All cell manipulations were carried out in an anaerobic glove box (Plas Labs Inc., MI, USA) with a $N_2/H_2/CO_2$ atmosphere (85/10/5 v/v). Cell suspensions were centrifuged outside the glove box for 5 min at 5,000 × *g* and 4°C, after the addition of sodium dithionite at a final concentration of 5.2 mg L^{-1} to ensure complete anaerobiosis. The cell pellets were resuspended in a reaction buffer (0.1 M Tris-HCl containing 40 mM NaCl and 5 mM $MgCl_2$; pH 7.2) [24] Cells were mixed with an equal volume of 0.1 mm silica beads and disrupted in a Mini-Beadbeater (BioSpec Products Inc., OK, USA) in 3 cycles of 20 s beating and 60 s cooling. Cell debris was removed by 5 min centrifugation at 12,000 × *g* (Minispin, Eppendorf, Hamburg, Germany) and the resulting CE was either used directly or stored under anaerobic conditions at -20°C until use. For determination of GAPDH kinetics, the CE was freed from compounds with a M_W below 5 kDa using a PD10 column (Sigma-Aldrich), as previously described [24].

Enzyme assays

All enzyme activity measurements were carried out as described previously [24], with the modification that 5.35 mM GAP was used in the assay for GAPDH activity. All assays were carried out in at least three technical replicates in the linear protein concentration range. In addition, the influence of the metabolites ATP, ADP and PPi on GAPDH activity in the concentration range of 1-10 mM was evaluated. The $K_{0.5}$ for the substrates GAP and NAD^+ were determined by using seven different GAP concentrations and varying the NAD^+ concentration. The *Ki* for NADH was determined by using four different NAD^+ concentrations and varying NADH concentration.

Background reactions for the assays were determined by replacing the substrate with the reaction buffer. One unit of enzyme activity (IU) is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per min. Specific activities are expressed as IU $(mg\ protein)^{-1}$. Protein concentration in the CE was determined according to Bradford (1976), with bovine serum albumin as a standard.

NAD(H)assay

The intracellular concentrations of NADH and NAD were determined by a cyclic assay as described earlier [25,24], with the exception of using phenazine ethosulfate (PES) instead of phenazine methosulfate (PMS), as PES is chemically more stable than PMS [25].

Measurement of ATP and PPi

Samples were collected in screw-cap microcentrifuge tubes containing ice-cold chloroform and immediately frozen into liquid nitrogen. Samples were stored at -80° C until further analysis. During the sample preparation cells were not separated from the medium to avoid the loss of ATP and/or PPi due to possible leakage during centrifugation, as previously observed by Bielen et al [26]. Moreover, assays were also done to estimate the levels of ATP and PPi in the growth medium. ATP and PPi were extracted from the cells by using the cell lysis buffer as described in the protocol provided with the ATP Bioluminescence assay kit HSII (Roche Molecular Biochemicals). ATP was measured using the ATP Bioluminescence assay kit HSII (Roche Molecular Biochemicals) containing luciferin/luciferase reagent, according to the protocol provided with the kit, in a tube-reading 1250 Luminometer (LKB-Wallac, Turku, Finland).

Samples for PPi measurement were treated with ATP-sulfurylase (Sigma-Aldrich, Germany) in the presence of excess Adenosine-5'-phosphosulfate to produce ATP from PPi [27] and the overall ATP was measured with the Bioluminescence assay kit HSII (Roche Molecular Biochemicals). Since significant amounts of ATP were present in the samples, the assay was started with the measurement of ATP, to convert most of the ATP into PPi and immediately ATP sulfurylase was added to convert overall PPi into ATP, which was subsequently measured. The background signal, less than 15% of the total signal in all measurements, was subtracted from the total signal to estimate the net PPi concentration. Intracellular levels of ATP and PPi were calculated as previously described [26].

Calculations

H_2 productivity (mM h^{-1}) and cumulative H_2 formation (CHF, mM) were calculated in two different ways depending on the experimental design. All calculations were based on the ideal gas law using H_2 and CO_2 concentration in the headspace. For *Case I* (sparging with N_2) the calculations were based on the flow rate of the influent N_2 gas and the percentages of H_2 and CO_2 in the effluent gas, as no other gases were detected, whereas for *Case II* (no sparging) the flow rate of the

effluent gas was measured by the water displacement method with CO_2-saturated water to avoid any further CO_2 to dissolve. It was assumed that CO_2 in the effluent gas did not dissolve in the CO_2-saturated water; therefore the actual dissolved CO_2 concentration was not determined. CO_2-saturated water was prepared by stripping the boiling water with 100% CO_2, cooling it down simultaneously and was kept cold throughout the experiment. At the steady state, the flow rate of the effluent gas was determined by measuring the volume of the effluent gas collected between two time points. Thus, H_2 productivity and CHF were calculated based on hydrogen concentration in the effluent gas and the flow rate of the effluent gas.

The intracellular specific productivities (mmol.g^{-1}.h^{-1}), i.e. q_{NADH} produced, q_{NADH} used, q_{NADH} available and $q_{pyruvate}$, were estimated as described previously [28].

The biomass yield per mol of ATP ($Y_{x/ATP}$; g.mol^{-1}) was calculated based on the equation previously described [15]:

$$Y_{x/ATB} = \frac{[\text{biomass}]}{1.5 \times [\text{acetate}] + 0.5 \times [\text{lactate}] + 0.5 \times [\text{ethanol}]} \quad (1)$$

The dissolved H_2 concentration ($H_{2,aq}$) in equilibrium was estimated according to Henry's law:

$$H_{2,aq} = P_{H2} \times K_H \quad (2)$$

where K_H is Henry's constant (mM/bar) and is dependent on the temperature according to:

$$\ln \frac{K_2}{K_1} = \frac{\Delta H^0}{R} * \left[\frac{1}{T_1} - \frac{1}{T_2} \right] \quad (3)$$

where K_1 is K_H at T_1 = 298 K (0.78 mM/bar, [29]), K_2 is the calculated K_H at T_2 = 343 K (0.52 mM/bar), ΔH (J/mol) is the enthalphy at standard conditions and R (8.314 J/mol/K) is the gas constant.

2.9 GAPDH model, data fitting and statistical analysis

The affinity constants for the substrates GAP and NAD^+ was determined by fitting the Michaelis-Menten type kinetic equation to the obtained data [30]:

$$v = V_{max} * \frac{[NAD^+] * [GAP]}{K_{GAP} * [NAD^+] + K_{NAD} * [GAP] + [GAP] * [NAD^+] + \alpha * K_{GAP}} \quad (4)$$

where V is the reaction rate, V_{max} is the maximum rate of the reaction, K_{GAP} and K_{NAD} are the affinity constants for GAP and NAD^+, respectively, and α is a constant representing any interaction between NAD and GAP binding to the enzyme. However, from analysis of our data α was not significantly higher than zero and was, therefore, the term "$\alpha \times K_{GAP}$" was excluded from the equation.

The inhibition kinetics of NADH was determined by using four different NAD^+ concentrations and varying NADH concentrations. The type of inhibition kinetics was visualized by fitting equations for i) sigmoidal competitive inhibition, ii) uncompetitive inhibition iii) mixed inhibition, or iv) linear competitive inhibition

$$v = \frac{V_{max} * (NAD^+) * (GAP)}{[K_{GAP} + (GAP)] * \left[K_{NAD} * \left(1 + \left(\frac{NADH}{K_{NADH}} \right) \right) + (NAD^+) \right]} \quad (5)$$

where K_{NADH} is an inhibition constant, to the experimental data [30].

Data obtained from the inhibition kinetics of NADH was also used to study the effect of NADH/NAD (redox ratio) on GAPDH activity by assuming redox ratio as a substrate. Hill type kinetic equation was fitted to the data [30]:

$$v = \frac{V_{max}}{1 + \left(\frac{K_R}{S} \right)^h} \quad (6)$$

where S is the redox ratio, K_R is an affinity constant and h is the Hill coefficient of cooperatively.

Parameter estimation (viz. V_{max}, α, K_{GAP}, K_{NAD}, K_{NADH}, K_R and h) was based on non-linear regression using the Surface Fitting Tool (sftool) or curve fitting tool (cftool) in MATLAB (R2009a), which also provides a statistical analysis. Model discrimination was based on the goodness of fit, which was evaluated by the 95% confidence bounds for the fitted parameters and by the square of the multiple correlation coefficients (R^2).

Estimations on LDH activity was based on previously published model of LDH regulation in *C. saccharolyticus* [24].

Results and discussion

3.1 Effect of P_{H2} on growth and lactate formation in batch cultures

C. saccharolyticus was cultivated in pH-controlled batch mode, with and without N_2 sparging. The P_{H2} peaked at 6.3 kPa with sparging and 67 kPa without sparging the culture, which allowed analyzing the influence of the P_{H2} on growth and product formation. In both cases, the organism grew at a similar rate until a critical P_{H2} of 11 kPa was reached in the gas phase of the non-sparged fermentor, at which the growth rate decreased by 24% (Figure 1A). Moreover, when the P_{H2} reached 30 kPa (after 14 h of growth; Figure 1A) growth became linear. In contrast, the sparged culture grew exponentially until glucose was almost depleted after 16 hours of incubation (Figure 1A and 1B). Inhibition of growth is probably a result of both high dissolved hydrogen

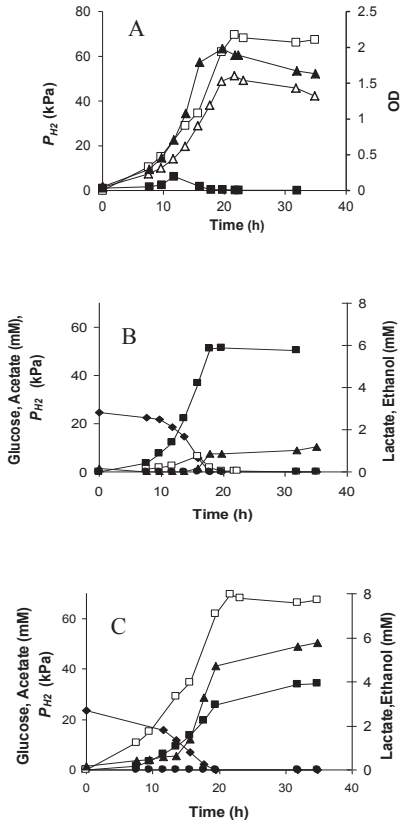

Figure 1 Growth and product formation by *C. saccharolyticus* in pH-controlled batch fermentations with and without N_2 sparging. (A) with sparging (filled symbols) and without sparging (open symbols) growth (▲, Δ) and P_{H2} (■, □). Product formation in the presence (B) and absence of N_2 sparging (C) glucose (◆), acetate (■), lactate (▲), ethanol (●) and P_{H2} (□). Note: Different scales are used on primary and secondary vertical axes.

concentration and high osmolarity due to high dissolved CO_2 concentrations in the non-stripped reactor [31].

Consistent with previous findings [24], acetate and H_2 were the main metabolic end products during exponential growth when the culture was sparged with N_2 (Figure 1B). Lactate formation was in this case initiated in the transition to stationary phase at a low P_{H2} (6.3 kPa).

Moreover, although some lactate was produced during early growth in the absence of sparging, the lactate productivity accelerated when the growth became linear after 14 hours of cultivation (Figure 1C). At this point, the P_{H2} was 30 kPa, thus about 2-fold higher than the previously quoted critical P_{H2} for lactate formation (10-20 kPa; [23]). The H_2 productivity was not determined due to technical difficulties in accurate determination of instable increasing productivities with water displacement techniques. These results indicated that in batch cultivations, the cells can withstand higher P_{H2} maintaining an exponential growth profile until lactate is started to be formed, accompanied with linear growth.

The increased P_{H2} clearly influenced the overall lactate yield, as the final lactate concentration was 5-fold higher when the culture was not sparged with N_2 (Figure 1B and 1C). The acetate/lactate ratio was 43 and 6 for the sparged and the non-sparged conditions, respectively. Ethanol, which acts as an alternative electron sink for *C. saccharolyticus*, was present in negligible quantities, irrespective of the P_{H2} (Figure 1B, C).

This behaviour is consistent with the outcome of another study on metabolic shifts in *C. saccharolyticus* [31], which demonstrated that a combination of the osmotic pressure and the dissolved H_2 concentration determines the metabolic shift to lactate production. The key players behind this are the intracellular energy carriers that influence the lactate dehydrogenase (LDH) [24]. The kinetics of LDH demonstrated that the anabolic byproduct and energy carrier, PPi, plays a central role in the allosteric regulation of this catabolic enzyme by acting as a strong competitive inhibitor (Ki = 1.7 mM), therefore antagonizing the stimulating effect of elevated NADH/NAD ratios [24]. The PPi levels in *C. saccharolyticus* are correlated to the growth rate, as the PPi concentration is highest (4 ± 2 mM) during exponential growth, and decreased seven folds during the transition to the stationary phase [26]. Consequently, at exponential growth, these high PPi levels assure that LDH remains inactive even at higher NADH/NAD (1.2 $mol.mol^{-1}$) ratios. However, when the growth rate decelerates the concomitant decrease in PPi levels enables LDH to become sensitive to an increase in the NADH/NAD ratio [24]. Therefore, lactate is not formed even at high P_{H2} (< 30 kPa), as long as the cells are able to sustain high PPi levels through maintaining a high growth rate.

Effect of P_{H2} on growth and lactate formation in continuous culture

Carbon-limited chemostat cultures were used to investigate the effect of P_{H2} on *C. saccharolyticus* at a controlled physiological state. In the absence of gas sparging, the P_{H2} reached 67 kPa after about 10 volume changes at D = 0.05 h^{-1} without any washout of the culture indicating that *C. saccharolyticus* can withstand higher P_{H2} than was previously reported [23]. A

noticeable effect of the elevated P_{H2} in this case was the redirection of the pyruvate flux, i.e. 38% and 16% of the flux at the pyruvate node was directed to lactate and ethanol, respectively, whereas 0.5% and 4.1% of corresponding fluxes were observed in sparged cultures at similar dilution rates (Table 1). Interestingly, the overall catabolic rate ($q_{glucose}$) was not reduced at this high P_{H2} (Table 1). However, a steady state could not be attained in the absence of N_2 sparging at a higher D (0.15 h^{-1}) and the culture washed out at P_{H2} of 67 kPa. These results are in line with previous findings of supersaturation of hydrogen around the cell surface at high productivities due to mass transfer limitation [31]. Under the assumption of an equilibrium between dissolved ($H_{2,aq}$) and gaseous H_2, the $H_{2,aq}$ should be 0.24 μM at 67 kPa in the headspace and 70°C (Eq. 2 and 3), hence well below the critical $H_{2,aq}$ concentration for growth ($H_{2,aq\ crit}$ = 2.2 mM; [31]. However, the actual concentration around the cell depends on the ratio of H_2 productivity/H_2 mass transfer rate [31]. Therefore, the observation that the cells washed out at high D (0.15 h^{-1}) but retained at low D (0.05 h^{-1}) strongly indicates that at a lower growth rate, the H_2 productivity is in the same range as compared to the mass transfer rate such that $H_{2,aq} < H_{2,aq\ crit}$. In contrast, at the higher growth rate, the hydrogen productivity exceeds the mass transfer rate by far, especially under non-sparging conditions, resulting in supersaturation of hydrogen and thus extensive growth inhibition [31].

Under N_2-sparging conditions, the dilution rate also had an effect on product distribution. At D = 0.15 h^{-1}, the H_2 yield was lower than at D = 0.05 h^{-1} (2.9 ± 0.2 mol/mol glucose and 3.48 ± 0.09 mol H_2/mol glucose, respectively), which agrees with the findings of de Vrije et al [9]. Moreover, only 86% of pyruvate flux was directed to acetate at D = 0.15 h^{-1}, compared to a 95% flux at D = 0.05 h^{-1}. In addition, as previously reported [9], more residual glucose was observed at higher D (Table 1).

The biomass yield of *C. saccharolyticus* at low P_{H2} is slightly higher than that in *Clostridium cellulolyticum* [32], but similar to that in *Thermoanaerobacter ethanolicus* [33], and significantly lower than that in *Cl. acetobutylicum* [34]. The increase in the energetic biomass yield of *C. saccharolyticus* at the higher D was also observed for *Cl. cellulolyticum* [32] and *T. ethanolicus* [33].

Level of key redox-related catabolic enzymes

The increase in P_{H2} appeared to enhance lactate formation in *C. saccharolyticus* (Table 1). Therefore, the activities of different catabolic dehydrogenase enzymes were determined in cells grown in continuous cultures (Table 2). The specific activity of GAPDH decreased about 60% with an increase in the growth rate. On the other hand, the opposite trend was observed in the specific activities of LDH and ADH. The level of specific LDH activity increased almost eight folds in absence of sparging, which corresponded well with the observed increase in the lactate flux (Table 1, 2). This correlation is comparable to the 2-fold increase in specific LDH activity and lactate flux during the transition to the stationary phase in batch cultures of *C. saccharolyticus* on 10 $g.L^{-1}$ glucose [24]. Similarly, ADH specific activity increased four folds in absence of sparging (Table 2) consistent with four-fold increase in the ethanol flux (Table 1). It has

Table 1 Fermentation data in continuous cultivations of *C.saccharolyticus* on glucose (5g.L^{-1}) at steady states of different dilution rates, with and without N_2 sparging.

Parameter	Results obtained with and without N_2 sparging at D (h^{-1}) of:		
	0.05 (100 mL/min N_2)	0.15 (100 mL/min N_2)	0.05 no stripping
Biomass conc. (g/L)	0.51 ± 0.02	0.61 ± 0.05	0.42 ± 0.01
Residual glucose conc. (mM)	0.05 ± 0.03	3 ± 2	0.3 ± 0.3
$q_{glucose}$ (mmol/g/h)	2.90 ± 0.09	6.2 ± 0.4	3.2 ± 0.4
$q_{pyruvate}$(mmol/g/h)	4.90 ± 0.19	9.55 ± 0.03	5.8 ± 0.7
q_{H2} (mmol/g/h)	10. 1 ± 0.4	18.0 ± 0.0	5.9 ± 0.6
Product yield (mol/mol)			
H_2	3.48 ± 0.09	2.9 ± 0.2	1.82 ± 0.03
Acetate	1.61 ± 0.03	1.32 ± 0.13	0.83 ± 0.02
Lactate	0.01 ± 0.00	0.01 ± 0.01	0.67 ± 0.01
Ethanol	0.07 ± 0.00	0.20 ± 0.03	0.28 ± 0.04
Biomass (g/mol)	17.3 ± 0.5	24.1 ± 1.5	16 ± 2
Y_{ATP} (g cells/mol ATP)	5.4 ± 0.2	9.1 ± 0.3	6.7 ± 0.6
Carbon recovery (%)	0.96 ± 0.01	0.93 ± 0.04	0.93 ± 0.02
Redox recovery (%)	0.98 ± 0.01	0.93 ± 0.03	0.97 ± 0.02

Table 2 Enzyme levels (IU.(mg protein^{-1})) of key catabolic redox-dependent enzymes at steady states at different dilution rates, i.e. GAPDH, ADH, LDH and estimated activity (% of potential activity) of LDH (LDHv) under physiological conditions (Tables 3, 4) based on previously described kinetic model [23], of *C. saccharolyticus* cultures in the presence and absence of N_2 sparging. Presented data is average of one biological and at least three technical replicates at a linear range.

Enzyme	Results obtained with and without N_2 sparging at D (h^{-1}) of:		
	0.05 (100 mL/min N_2)	0.15 (100 mL/min N_2)	0.05 no sparging
GAPDH	3.5	1.4	1.3
ADH	0.44	0.96	1.8
LDH	1.2	2.6	8.3
LDHv	10	25	100

been shown previously that the levels of this enzyme in *C. saccharolyticus* increased three folds during batch growth at the onset of the stationary phase [24]. The levels of the GAPDH and ADH in *C. saccharolyticus* under N_2 sparging were comparable to the measured levels of the corresponding enzymes in *Cl. cellulolyticum* under equivalent conditions [32]. However, LDH activity was about three folds higher in sparged cultures of *Cl. cellulolyticum* at the low dilution rate [32], which could be a consequence of the strong regulation of the enzyme [24].

Inhibition of GAPDH by NADH

To investigate the effect of increased dissolved hydrogen concentration on the glycolytic flux, the effect of NAD and NADH on GAPDH activity in *C. saccharolyticus* was investigated *in vitro*. Conversion of GAP and NAD by GAPDH followed Michaelis-Menten kinetics, with $K_{0.5}$ values of 1.5 ± 0.3 and 0.28 ± 0.06 mM for GAP and NAD, respectively. Fitting the kinetic model to the data (Eq. 6) showed that there is no interaction between NAD and GAP when binding to the enzyme. NADH inhibited the reaction in a competitive manner (K_{NADH} = 0.03 ± 0.01 mM; Eq. 7), based on Dixon and Cornish-Bowden plots (Figure 2A and 2B) as well as through model discrimination by comparative fitting different inhibition models to the data (R^2 = 0.974 for the competitive model; data not shown). The activity of GAPDH appeared to follow Hill kinetics with respect to changes in NADH/NAD ratio (Figure 2C; R^2 = 0.9817; K_R = 0.09 ± 0.01; h = -0.8 ± 0.1). Moreover, increase in the NADH/NAD ratio up to one, was unable to fully inhibit the enzyme (Figure 2C). Based on the estimated value for *K*i of NADH, GAPDH in *C. saccharolyticus* was more resistant to increased NADH levels than the GAPDH of most other related bacteria.

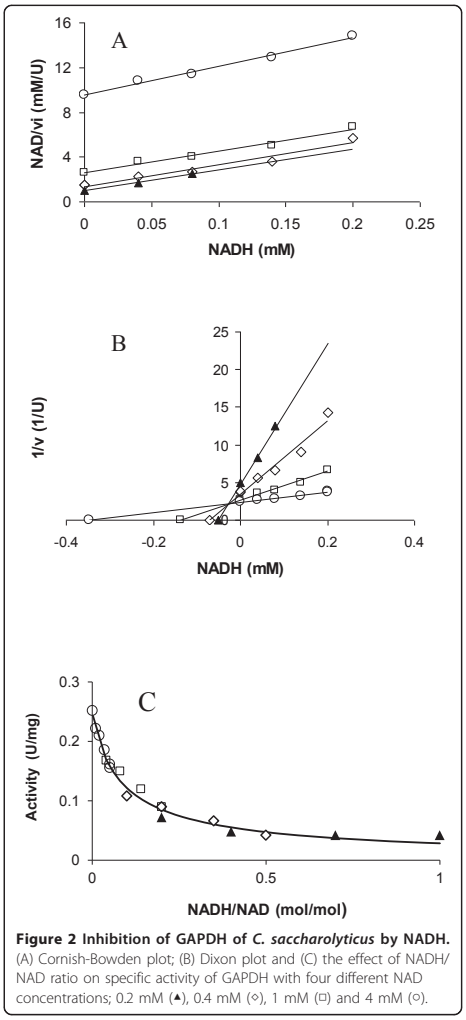

Figure 2 Inhibition of GAPDH of *C. saccharolyticus* by NADH. (A) Cornish-Bowden plot; (B) Dixon plot and (C) the effect of NADH/NAD ratio on specific activity of GAPDH with four different NAD concentrations; 0.2 mM (▲), 0.4 mM (◇), 1 mM (□) and 4 mM (○).

The NADH concentration that causes 50% inhibition of the enzyme of *C. saccharolyticus* was 0.03 mM, as compared to 0.01 mM for *T. thermohydrosulfuricus* (formerly known as *Clostridium thermohydrosulfuricum*; [35]) and *Cl. acetobutylicum* [34]. However, the enzyme of *C. saccharolyticus* is less resistant to NADH inhibition than that of *Cl. cellulyticum* [36], for which 50% inhibition was observed at 0.1 mM NADH (Figure 2C). Consistently, the NADH/NAD ratio in the cells of

Cl. cellulyticum is significantly higher than in *C. saccharolyticus* [24,37].

Finally, the activity of GAPDH was not found to be influenced by ATP, ADP or PPi over the physiological range of metabolite concentrations (1-10 mM).

Redox metabolism and its effect on the glycolytic flux

High P_{H2} can potentially inhibit H_2 formation through product inhibition of the hydrogenase-catalyzed NADH oxidation [38], which could lead to increased NADH/NAD ratios [37]. To investigate whether this was the case for *C. saccharolyticus* in the absence of sparging, NADH and NAD levels were determined at steady state conditions with and without sparging (Table 3). Interestingly, the NADH/NAD ratio remained similar (0.12 mol/mol; Table 3) at which the GAPDH activity was decreased for about 50% by NADH inhibition (Figure 2C). These results indicate that *C. saccharolyticus* sustains the NADH/NAD ratio at a homeostatic level as to support a high glycolytic flux. This could be achieved inside the cells by two mechanisms, i) by regulating the activity of GAPDH and/or ii) by redirecting pyruvate flux to more reduced products.

Under given conditions, the overall glycolytic flux can be measured as pyruvate flux. Hence, it could be argued that, glycolytic flux is a function of the activity of GAPDH ($q_{pyruvate} = f(v_{GAPDH})$). The activity of GAPDH, in turn, is a function of NADH/NAD ratio ($v_{GAPDH} = f$ (NADH/NAD); Figure 2C; Figure 3). Consistently, a slight decrease in the NADH/NAD ratio appears to result in 94% higher pyruvate flux as a consequence of 9% increase in the GAPDH activity (at D = 0.15h^{-1}; Table 1, 2; Figure 2C).

At high P_{H2}, 53% of the pyruvate flux was redirected to lactate and ethanol (Table 1). This redirection is clearly illustrated by the estimated ratio of the NADH production flux over the NADH flux to reduced products (q_{NADH}produced/q_{NADH}used), which is eight folds lower at high P_{H2} (Table 3). At this higher flux to reduced products, the NADH available for hydrogen formation is decreased (2.5 folds) as a strategy of the cell to maintain low NADH/NAD ratios at all the conditions,

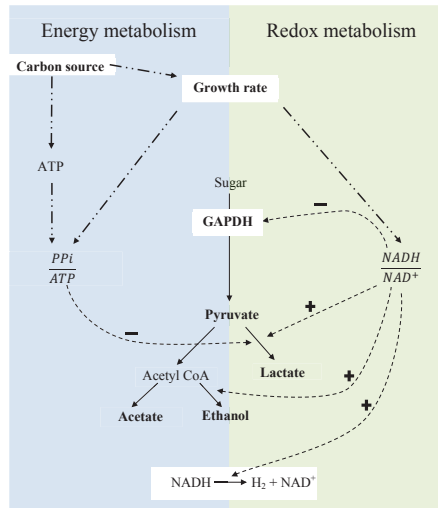

Figure 3 Summary of the various factors affecting H_2 tolerance in *C. saccharolyticus*. Regulation at enzyme level is depicted by the 'simple dashed' line and at metabolic level by 'dash-dotted' line; 'plus' sign shows activation and 'minus' sign shows inhibition.

including where the elevated dissolved hydrogen concentration inhibits the NADH-dependent hydrogenase reaction. This inhibition of hydrogen formation is evaluated by thermodynamic analysis.

Thermodynamic analysis

The critical P_{H2} is dependent on the NAD/NADH ratio and the temperature according to (Eq. 7, [39,40]):

$$P_{H2} = \frac{[NAD(P)H]}{NAD(P)^+} e^{-\left[\frac{E'^0 NAD(P)^+ - E'^0 H_2}{\frac{RT}{2F}}\right]} \quad (7)$$

Table 3 Measured NADH/NAD ratios and NADH concentration and estimated redox fluxes at steady states of different dilution rates of *C.saccharolyticus* cultures in the presence and absence of N_2 sparging

Parameter	Results obtained with and without N_2 sparging at D (h^{-1}) of:		
	0.05 (100 mL/min N_2)	0.15 (100 mL/min N_2)	0.05 no stripping
NADH/NAD (mol/mol)	0.13 ± 0.02	0.10 ± 0.00	0.12 ± 0.00
q_{NADH} produced (mmol/g/h)	4.90 ± 0.2	9.55 ± 0.03	5.8 ± 0.7
q_{NADH} used (mmol/g/h)	0.44 ± 0.03	2.6 ± 0.5	4.0 ± 0.7
q_{NADH} produced/q_{NADH} used (mol/mol)	11	3.7	1.4
NADH available for H_2:ase (mmol/g/h)	4.5	7.0	1.8

where E° is the midpoint reduction potential, F is the Faraday's constant, R is the ideal gas constant and T is the absolute temperature (K).

Therefore, lower NADH/NAD ratios make the hydrogenase reaction more energetically favourable, which was confirmed experimentally by Veit et al [39]. Consistently, the ethanol-adapted *T. thermohydrosulfuricus* strain, which is more tolerant to H_2 than the wild-type strain, possessed a GAPDH which could tolerate approximately twice the amount of NADH concentrations as the GAPDH of its wild-type variant [35].

At NADH/NAD ratios of 0.12 mol/mol (Table 3) measured herein, the critical P_{H2} for hydrogen production at 70°C should be 12 Pa in the headspace (Eq. 7) and thus H_2-generation should not be possible to occur spontaneously at 67 kPa. Yet, *C. saccharolyticus* cultures were able to produce H_2 at this high P_{H2}.

One way to circumvent this apparent contradiction could be via the substrate specificity of different hydrogenase enzymes. Based on sequence similarity, *C. saccharolyticus* possesses two distinct hydrogenases, one NADH-dependent Fe-only hydrogenase (Csac_1860-1864) and one ferredoxin (Fd)-dependent, membrane-associated NiFe-hydrogenase (Csac_1540-1545; [17]). Given that the redox potential of the Fd couple (Fd_{red}/Fd_{ox}) is close to that of H_2 (approx. -400 mV, albeit depending on the involved enzyme [41]), the reaction is energetically favourable even at P_{H2} close to 39 kPa. Therefore, it is possible that the NiFe hydrogenase-catalyzed reaction in *C. saccharolyticus* is still functioning even at elevated dissolved H_2 concentrations. It is noteworthy that the NADH-dependent Fe-only hydrogenase in *T. tengcongensis* was down regulated at high P_{H2}, while the Fd-dependent hydrogenases were constitutively expressed, irrespective of the P_{H2} [42].

An alternative explanation can be proposed related to the finding of Schut and Adams [43], concerning the Fe-only hydrogenase in *T. maritima* using NADH and Fd_{red} simultaneously in a bifurcating manner. This novel bifurcating hydrogenase could therefore catalyze the unfavourable oxidation of NADH to H_2 by using the exothermic oxidation of Fd_{red} to drive the reaction. It is noteworthy that the sequence of the Fe-only hydrogenase in *C. saccharolyticus* (Csac_1860-1864) is similar to the bifurcating hydrogenase in *T. maritima* (TM1424-TM1426 [43]). However, it remains to be investigated whether this hydrogenase enzyme in *C. saccharolyticus* possesses a bifurcating function.

Energy metabolism and its impact on lactate and hydrogen formation

The total ATP and PPi pool in *C. saccharolyticus* increased with the growth rate (Table 4), which is in contrast to what was reported for ATP for *C. cellulolyticum* [32]. In addition, increased levels of both ATP and PPi were observed in the absence of sparging (Table 4) probably due to cell lysis caused by high dissolved CO_2 concentrations as ATP and PPi were released in the culture broth [15,31]. The latter was confirmed by lower biomass concentration (Table 1). The levels of ATP and PPi are in the same range as under stationary growth of *C. saccharolyticus* [24] and the PPi/ATP ratios are low in all conditions (Table 4). This suggests that LDH should be present in an active configuration during all conditions. However, due to the low NADH/NAD ratio of 0.1 mol/mol, the sensitivity of LDH to changes in PPi/ATP ratio is stronger [24]. In addition, the level of LDH depends on the cultivation condition (Table 2). Consequently, the slightly higher PPi/ATP ratio reduces the estimated activity of LDH 10 and 2.5 folds at low D and sparged conditions compared to the non-sparged and high D condition, respectively (Table 2, LDHv), partly explaining the significantly lower lactate yields in these conditions. However, the overall glycolytic and shifts in by-product formation is complex and merits more in depth studies. In addition, there is a competition for pyruvate at the pyruvate node (Table 1). Therefore, LDH kinetics alone could not explain the insignificant amount of lactate formed at higher D (0.15 h^{-1}) even at higher measured LDH activity in this condition (Table 1 and 4).

Effect of the carbon source on H_2 tolerance

The H_2 tolerance in *C. saccharolyticus* is not only dependent on the growth phase of the organism (Figure

Table 4 ATP and PPi levels at steady states of different dilution rates of *C.saccharolyticus* cultures in the presence and absence of N_2 sparging

Parameter	Results obtained with or without stripping and at D (h^{-1}) of:		
	0.05 (100 mL/min N_2)	0.15 (100 mL/min N_2)	0.05 no stripping
ATP mM	0.67 ± 0.07	0.80 ± 0.06	2.0 ± 0.2
ATP μmoles/g of cells	3.06 ± 0.32	3.66 ± 0.26	8.95 ± 0.91
PPi mM	0.92 ± 0.05	0.81 ± 0.07	2.1 ± 0.4
PPi μmoles/g of cells	4.20 ± 0.22	3.69 ± 0.34	9.62 ± 1.69
PPi/ATP	1.37	1.01	1.05

1). The results obtained in this study indicated that the critical P_{H2} for initiating lactate formation of *C. saccharolyticus* when grown on glucose was significantly higher than that of previously reported when the organism was grown on sucrose [23]. This led us to investigate whether various carbon sources would allow *C. saccharolyticus* to possess different H_2 tolerances in an experimental set-up similar to that used by van Niel et al [23]. The fermentations were carried out in batch mode and the gas outlet of the bioreactor was closed at the beginning of the lag phase, leading to a build-up of the total pressure in the vessel due to accumulation of H_2 and CO_2. Indeed, the acetate and lactate fluxes were considerably influenced by the carbon source. For instance, the acetate/lactate ratio was 6 in the experiments on xylose, which can be compared to the significantly lower acetate/lactate ratio of 0.26 previously observed on sucrose [23]. The acetate and the lactate yield were 1.2 and 0.21 mol/mol C6, respectively on xylose. In addition, lactate formation remained low in cultures on xylose, during the entire time span and acetate production still continued at P_{H2} up to 60 kPa (data not shown), while lactate was formed on sucrose when H_2 accumulated beyond 10-20 kPa [23]. Every mol of lactate formed deprives the cells not only of a mol of H_2 but also from obtaining an extra ATP. This is in accordance with previous work showing how the glycolytic flux is significantly increased when xylose is used as carbon source compared to that of sucrose [44]. High throughput technologies can be used to investigate this further in the future. Thus, the observed variability in the extent and sensitivity of lactate formation is also related to the energy metabolism of the cells that may vary with each carbon source (Figure 3; [11]).

Conclusions

C. saccharolyticus has the attractive property of producing high H_2 yields under ideal conditions. When the P_{H2} rises it has the ability to maintain glycolytic flux by regulating GAPDH. Required GAPDH activity is attained by keeping the NADH/NAD ratio relatively low through redistributing its metabolism towards more reduced end products, including lactate and ethanol. The results herein reveal that these redistributions are not solely dependent on the P_{H2}, but also on the growth state of the organism and the carbon source fermented. Although ethanol is produced, lactate remains the main alternative for *C. saccharolyticus* for reoxidizing NADH. For an economically attractive industrial application of *C. saccharolyticus*, hydrogen yields need to be kept maximized, for which metabolic shift to lactate should be kept at bay. In addition, H_2 production should be achieved preferably without the need for sparging gas to prevent central costs for the gas-upgrading process [45]. A critical P_{H2} should be set so as not to adversely affect the growth rate or biomass yield of the organism or to enhance lactate formation. This should be combined with a careful selection of the feedstock, based on the type of substrates, and operating at adequately low osmotic pressures [15]. Thus, according to our results, a xylose-rich feedstock is preferred over a sucrose-rich one, since the latter enforces an earlier effect of P_{H2} on growth and lactate formation than the former. If H_2 production is possible on a xylose-rich lignocellulosic feedstock and accomplished at high yields at high P_{H2}, it is definitely a critical step further towards a cost-effective biohydrogen process.

Abbreviations

D: dilution rate; h^{-1}; $q_{acetate}$: specific formation/consumption rate;mmol $(gCDW)^{-1}$ h^{-1}; $q_{ethanol}$: specific formation rate of ethanol; mmol $(gCDW)^{-1}$ h^{-1}; $q_{lactate}$: specific formation rate of lactate; mmol $(gCDW)^{-1}$ h^{-1}; q_{NADH}: produced specific formation rate of NADH; mmol $(gCDW)^{-1}$ h^{-1}; q_{NADH}: used specific formation rate of NADH used for lactate and ethanol; mmol $(gCDW)^{-1}$ h^{-1}; $q_{glucose}$: specific consumption rate of glucose; mmol $(gCDW)^{-1}$ h^{-1}; $q_{pyruvate}$: specific formation rate of intracellular pyruvate; mmol $(gCDW)^{-1}$ h^{-1}; $Y_{x/ATP}$: energetic biomass yield; gCDW molATP^{-1}; μ: specific growth rate; h^{-1}; P_{H2}: partial H_2 pressure; kPa; K_i: inhibition constant

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Authors' contributions

KW planned the content of the article. KW also planned and preformed the batch experiments and was involved in the planning of the continuous cultures experiments and enzyme kinetics experiments. SP planned and performed the continuous cultures, enzyme kinetics and metabolite analysis. KW and SP both wrote a part of the paper. EvN was involved in the planning of the experiments and supervised the processes. EvN also critically reviewed the text. All authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Biohydrogen production from wheat straw hydrolysate using *Caldicellulosiruptor saccharolyticus* followed by biogas production in a two-step uncoupled process☆

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ABSTRACT

A two-step, un-coupled process producing hydrogen (H_2) from wheat straw using *Caldicellulosiruptor saccharolyticus* in a 'Continuously stirred tank reactor' (CSTR) followed by anaerobic digestion of its effluent to produce methane (CH_4) was investigated. *C. saccharolyticus* was able to convert wheat straw hydrolysate to hydrogen at maximum production rate of approximately 5.2 L H_2/L/Day. The organic compounds in the effluent collected from the CSTR were successfully converted to CH_4 through anaerobic digestion performed in an 'Up-flow anaerobic sludge bioreactor' (UASB) reactor at a maximum production rate of 2.6 L CH_4/L/day. The maximum energy output of the process (10.9 kJ/g of straw) was about 57% of the total energy, and 67% of the energy contributed by the sugar fraction, contained in the wheat straw. Sparging the hydrogenogenic CSTR with the flue gas of the UASB reactor ((60% v/v) CH_4 and (40% v/v) CO_2) decreased the H_2 production rate by 44%, which was due to the significant presence of CO_2. The presence of CH_4 alone, like N_2, was indifferent to growth and H_2 production by *C. saccharolyticus*. Hence, sparging with upgraded CH_4 would guarantee successful hydrogen production from lignocellulosic biomass prior to anaerobic digestion and thus, reasonably high conversion efficiency can be achieved.

1. Introduction

Foreseeing the advancements in the energy infrastructure and end-user technologies, the growth in world energy consumption can be expected to slow down [1]. However, the supply of fossil fuels is expected to hit rock-bottom in coming decades [1]. Moreover, un-restrained usage of fossil fuels has contributed to growing concern over global warming. Hence, it

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is more than evident that the world needs alternative, renewable energy sources which should also be environmental friendly.

Of late, agricultural residues are increasingly being considered as a potential source of renewable biomass. Estimations of agricultural residues are about 10^{10} tons/year globally, corresponding to 4.7×10^{10} GJ of energy (about 9% of the global energy consumption in 2008 [1]), and about two-thirds consists of cereal residues [2]. Wheat straw is a lignocellulosic biomass, consisting of 35–40% cellulose, 20–30% hemicelluloses and 8–15% lignin [3]. These sugars can potentially be used in microbial fermentations to produce biofuels, such as, bioethanol, biogas and hydrogen. So far, however, bioethanol production from lignocellulosic biomass has not been successful enough due to a variety of techno-economic challenges [4–6]. Alternatively, studies have shown efficient production of hydrogen (H_2) from wheat straw hydrolysate (WSH) by dark fermentation (DF) [7–9]. H_2 is widely considered as a fuel of the future due to its properties of rapid burning speed, no emissions of greenhouse gases, higher energy density, low minimum ignition energy and a very high research octane number [10–13]. Currently, H_2 is mainly produced by reforming fossil fuels making it a non-renewable and non-carbon neutral, which is in contrast to what DF of agricultural residues has to offer. The thermophilic *Caldicellulosiruptor saccharolyticus* possesses the ability of producing H_2 via DF at yields near the theoretical maximum of 4 mol H_2/mol of hexose consumed [14]. In addition, *C. saccharolyticus* can naturally ferment a wide range of poly-, oligo- and mono-saccharides including sugars present in lignocellulosic hydrolysate [15]. Moreover, the absence of 'carbon catabolite repression' enables it to co-ferment glucose, xylose and arabinose among other sugars [16].

During the DF, the highest theoretical maximum yield of H_2 can be obtained only when acetate is the major by-product [17]. The latter, contains as much as 67% of the total energy present in the substrate. This energy can be retrieved in the form of H_2 by either photo-biological process or microbial electrolysis, which are both, however, still under development [18]. Alternatively, the effluent from DF can be transferred to an anaerobic digester, wherein acetate can be converted to CH_4 by acetoclastic methanogenesis, which is a reliable and an industrially established process [3,18]. Various studies of combined H_2 and CH_4 production in a two-step process have been reported in recent years [9,19]. Furthermore, H_2 and CH_4 together can give a mixture termed *hythane*, which has superior combustion properties compared to CH_4 alone [20].

So far, DF has been carried out largely in a continuously stirred tank reactor (CSTR), in which sparging is needed to actively remove hydrogen to keep the hydrogen partial pressure (p_{H_2}) to a minimum [21,22]. Nitrogen is usually used for sparging at lab-scale, as it is a cheap and inert gas. However, separation of N_2 from H_2 is tedious and thus not exploitable at industrial scale. As an alternative, CO_2 is relatively easier to separate from H_2, but has a detrimental effect on growth of *C. saccharolyticus* [23]. Finally, the CH_4 produced in the anaerobic digestion (AD) can, in principle, be used as sparging gas in the DF, producing *hythane*, after removal of CO_2.

The ability of *C. saccharolyticus* to ferment wheat straw was observed previously [7]. However, since the experiments were performed on raw wheat straw, they were continued for long duration (about 45 days [7]), which makes it economically unfeasible. On the other hand, various pretreatment methods can generate by-products which may inhibit microbial growth [24,25]. Hence, in this study, we demonstrate the fermentability of pre-treated wheat straw by *C. saccharolyticus* and its ability to sustain growth in the presence of CH_4. We also demonstrate the feasibility of the two-step process, wherein, the wheat straw hydrolysate (WSH) is fermented to produce H_2 in a CSTR by *C. saccharolyticus* and the effluent produced is converted to CH_4 by methanogens in a UASB reactor. During this study, the reactors performing DF and AD were uncoupled. Ideally, however, both the reactors should be coupled together as described previously [26].

2. Materials and methods

2.1. Wheat straw hydrolysate

WSH was produced by steam acid pretreatment and enzymatic hydrolysis of wheat straw obtaining an energy content of 11.9 MJ/kg of dry matter (DM) in the WSH. Glucose and xylose were the main sugars and the chemical oxygen demand (COD) was estimated to be 196 g/l. The detailed composition of the hydrolysate has been reported previously [27]. The pre-treated hydrolysate was centrifuged for 15 min at 4900 rpm to remove any remaining solid matter. Subsequently, the supernatant is then allowed to pass through a Whatman's no.1 filter paper supported by a nylon membrane to get rid of insoluble particulate matter. The pH of this clarified hydrolysate was adjusted to pH 7 with 12.5 M NaOH. The filtered neutral hydrolysate was sterilized by filtration using disposable Acrocap™ (pore size – 0.2 μm) filters and the filtrate was collected in sterile screw cap bottles and stored at −20 °C until further use.

2.2. Microorganism and culture medium

C. saccharolyticus DSM 8903 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). A modified DSM 640 medium was used as a base medium for all cultivations throughout this work [23]. Routine subcultures and inoculum development were conducted in 250 mL serum bottles containing 50 mL of medium under a N_2 atmosphere. Anoxic solutions of glucose, xylose and arabinose were autoclaved separately and were added to the sterile medium at the required concentration. Filter sterilized WSH was added to a sterile serum bottle and was kept under a N_2 atmosphere.

2.3. Experimental set-up and operation

Batch cultures of dark fermentation were carried out at 70 °C using 250-mL serum flasks containing 50 mL liquid medium. The preparation of anaerobic flasks was as follows: the modified DSM 640 medium without the carbon source was added to the flasks and thereafter, the flasks were sealed with butyl stoppers and aluminium crimps. Subsequently, the headspace of the flasks was flushed with N_2 unless stated

otherwise. Two separate batch tests were performed: *a*) fermentability test of WSH and *b*) effect of CH_4 present in the headspace on the growth of *C. saccharolyticus*. In the former, four different concentrations of hydrolysate (v/v), 20%, 10%, 6.66% and 5%, were studied. Flasks containing 6.66% and 5% hydrolysate were complimented with pure sugars (glucose, xylose and arabinose) to keep the total sugar concentrations at the level present in 10% v/v WSH (i.e. in g/L glucose, 6.7, xylose, 3.7, and arabinose, 0.4). In test '*b*', the headspace of the flasks was flushed with either CH_4 or N_2. 10% v/v of hydrolysate was used as substrate and a medium with pure sugars was used as control. During all batch experiments, samples were collected at regular time intervals for the determination of biomass, H_2 accumulation and metabolite concentrations. Experiments were continued until H_2 accumulation ceased in the headspace.

The chemostat cultures were carried out as described previously [22] except for the following modifications. In continuous mode, the reactor was fed with a fresh medium containing (per litre of deionised water) NH_4Cl 0.9 g, $MgCl_2.7H_2O$ 0.4 g, KH_2PO_4 0.75 g, K_2HPO_4 1.5 g, Yeast extract 1 g, resazurin 1 mg, trace element solution SL-10 [28] 1 mL and WSH (10% v/v) as a substrate but omitting cysteine-HCl. WSH at 10% v/v contained approximately 11 g/L of total monosaccharide sugars with 23 mg/L of 5-(hydroxymethyl)furfural (HMF) and 114 mg/L of furfural [27]. The reactor was sparged with either 100% N_2 or a gas mixture containing $N_2 + CO_2$ (60%:40% v/v) at the flow rate of 6 L/h. The steady states were obtained at four different conditions, i.e. *Case I*, low growth rate ($D = 0.05\ h^{-1}$), N_2 sparging; *Case II*, higher growth rate ($D = 0.15\ h^{-1}$), N_2 sparging; *Case III*, low growth rate ($D = 0.05\ h^{-1}$), sparging with a mixture of N_2 (60% v/v) and CO_2 (40% v/v); and *Case IV*, higher growth rate ($D = 0.15\ h^{-1}$), sparging with a mixture of N_2 (60% v/v) and CO_2 (40% v/v). The steady states were determined after at least five volume changes based on the stability of CO_2 and H_2 levels and biomass concentration. The effluent generated from the chemostat was collected, mixed together and stored at 4 °C before use in AD.

Batch cultures of AD were performed in triplicates using the effluent from DF. The flasks were incubated at 37 °C for 31 days. The experimental procedure and set-up was as described earlier [27,29]. Methane production using the effluent of dark fermentation was performed in UASB reactors in duplicate and under mesophilic (37 °C) conditions. The active reactor volume was 0.8 L and the up-flow velocity was 0.08 and 0.09 mL/h. The rest of the reactor configuration was as previously described [30]. A modified basic anaerobic nutrient solution (BA) was used to supplement the effluent [31], in that, ammonium chloride was substituted with Urea (1 g/L), as the latter is a rich nitrogen source and also a buffering agent. The effluent collected from DF had a pH of 6.6 and a COD of 16.2 g/l before addition of the BA medium. After addition of the BA medium, the pH and the COD changed to 6.9 and 15.3 g/l, respectively (Table 2). Prior to the treatment of the DF effluent, the UASB reactor was continuously fed with the WSH containing about 10 g/l of fermentable sugars. When the feed was switched to DF effluent, the reactors were operated at an OLR of 5.0 g COD/L/day (HRT of 2 days) until they reached stability. Increase in the organic loading rate was performed by decreasing the hydraulic retention time (HRT). The HRT was decreased from 2.5 to 1.5 days and corresponded to an increase in OLR of 6.0–10.5 g COD/L/day. The treatment period was 49 days.

2.4. Analytical methods

For dark fermentation, gas in the headspace of the serum flasks and the CSTR was analysed for CO_2 and H_2 by gas chromatography, using a dual channel Micro-GC (CP-4900; Varian gas chromatography, Middelburg, The Netherlands), as previously described [28]. The results were analysed with a Galaxie Chromatography workstation (v 1.9.3.2). The optical density of the culture was measured at 620 nm using a U-1000 spectrophotometer (Hitachi, Tokyo, Japan). The cell-free culture medium was used as a blank while measuring the optical density of the cultures. The cell dry weight was determined as previously described [32]. The metabolites, sugars, 5-(hydroxymethyl)furfural and furfural in DF were analysed by HPLC (Waters, Milford, MA, USA) as described previously [22].

The samples collected during anaerobic digestion were analyzed for pH, COD, NH_4^+–N, partial and total alkalinity, volatile fatty acids, gas volume and composition. Methods of sample collection and analysis for the methane potential batch test and UASB reactor were as previously described [27]. The volume of methane and hydrogen were corrected for using the standard conditions (0 °C, 1 atm).

2.5. Calculations

The volumetric H_2 productivity (mM/h) was calculated using the ideal gas law and the H_2 and CO_2 concentrations in the headspace of the serum flasks or CSTR. In case of the CSTR, the calculations were based on the flow rate of the effluent gas and the accompanying partial pressures of H_2 and CO_2. In case of serum flasks, the product gas was allowed to accumulate in the headspace, which is the basis for the calculation. The energy output for each of the cases was calculated based on lower calorific values (LCV) and the quantity of H_2 or CH_4 produced. The LCV for H_2 and CH_4 are 122 and 50.1 MJ/kg, respectively [33].

3. Results

3.1. Fermentability of wheat straw hydrolysate in DF

Media containing 10% or lower levels of WSH showed comparable biomass and H_2 yields, (Fig. 1(A)). Even though, the differences observed were insignificant, yet a decreasing trend can be observed in maximum obtainable H_2 productivities with increasing WSH concentration (Fig. 1(A)). Hardly any or no significant growth and H_2 accumulation was observed in the flasks containing 20% WSH (data not shown). Interestingly, H_2 accumulation and cell growth appears to be enhanced in WSH compared to a medium with only pure sugars (Figs. 1(A) and 2). For obvious reasons, 10% v/v of WSH was added in a growth medium used in further experiments.

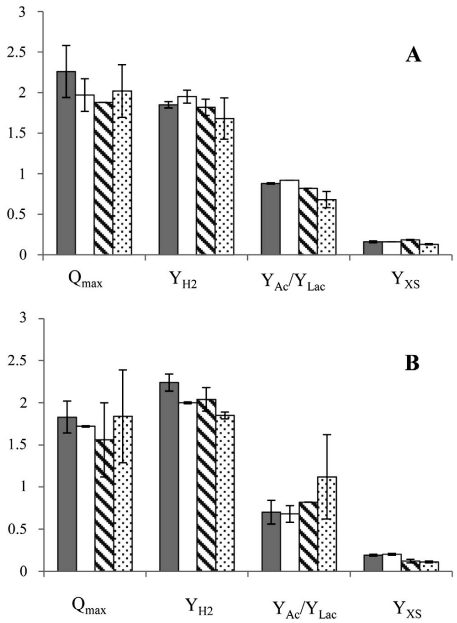

Fig. 1 – Results of the batch fermentations in DF to evaluate the fermentability of WSH by *C. saccharolyticus*. Q_{max} – maximum H_2 productivity (mmol/L/h); Y_{H_2} – hydrogen yield (mol/mol glucose consumed). Y_{Ac}/Y_{Lac} – the ratio of acetate yield to lactate yield; Y_{xS} – the biomass yield (mol/mol glucose consumed). (A) Fermentability of WSH (v/v), 5% (■), 6.67% (□), 10% (▨) and pure sugars (▩). (B) Fermentability of 10% (v/v) WSH in presence of either CH_4 or N_2 in the headspace, 10% WSH + CH_4 (■), 10% WSH + N_2 (□), Pure sugars (glucose, xylose and arabinose) + CH_4 (▨) and Pure sugars + N_2 (▩).

3.2. *Growth of C.* saccharolyticus *in presence of methane*

H_2 productivities and biomass yield seemed to be unaffected by CH_4 (Figs. 1(B) and 2). Interestingly, the flasks containing 100% CH_4 in the headspace appeared to have slightly higher H_2 yields compared to those containing 100% N_2 in the headspace (Figs. 1(B) and 2). Yet again, the flasks containing WSH showed relatively better biomass formation and H_2 accumulation at a higher maximum growth rate than those containing only pure sugars (Figs. 1(B) and 2). All batch experiments displayed co-consumption of glucose, xylose and arabinose. However, xylose was the most preferred substrate regardless of the growth conditions (Fig. 2).

Although, CH_4 is slightly beneficial; for safety reasons, N_2 was used in all following experiments instead, as both do not affect the performance of *C. saccharolyticus* negatively. Thus, the gas mixture of $N_2 + CO_2$ was assumed to mimic the non-upgraded flue gas ($CH_4 + CO_2$) from the AD (*Case III* and *IV*). Similarly, cultures sparged with N_2 were assumed to be the same as if sparged with CH_4 (*Case I* and *II*).

3.3. *Growth of C.* saccharolyticus *on WSH in controlled bioreactors*

In chemostats, four different experimental conditions were employed (using the growth rate and sparging gas composition as variables, *Cases I to IV*), with a medium containing 10% WSH as carbon source. Out of the four conditions studied, a low growth rate ($D = 0.05\ h^{-1}$) and sparging the reactor with N_2 resulted in the highest H_2 yield and best of substrate conversions (Table 1). The substrate conversion efficiency decreased with increasing growth rate and when CO_2 was present in the sparging gas. Surprisingly, at a higher growth rate ($D = 0.15\ h^{-1}$), the culture sparged with $N_2 + CO_2$ displayed a higher H_2 yield and higher specific H_2 production rate than the one sparged with N_2 (Table 1). Also, the highest lactate yield per mole of hexose was observed in the latter case compared to the other conditions. However, the average volumetric H_2 productivity was about 40% higher in the reactors sparged with N_2 only (Table 1, 5.1 L H_2/L/day) than the reactors sparged with $N_2 + CO_2$ (Table 1, 2.9 L H_2/L/day). The overall conversion of substrate in the dark fermentation was found to be in the range of 19–88% (Table 1). Regardless of the growth conditions the culture was able to reduce the potential growth inhibitors (5-(hydroxymethyl)furfural and furfural) present in the WSH (Table 1). Cultures sparged with $N_2 + CO_2$ displayed higher medium osmolalities than their counterparts performed with N_2 sparging (Table 1). Similarly, low amounts of biomass were obtained in chemostats sparged with $N_2 + CO_2$ which were accompanied by higher amounts of residual sugars and consequently lower conversions. The specific consumption rate for xylose was significantly higher than that for glucose in the cultures sparged with $N_2 + CO_2$ (*Case III* and *IV*, Table 1), whereas the opposite was true for the cultures sparged with N_2 (*Case I* and *II*, Table 1). Carbon and redox recovery was significantly higher than 100% in all the cases studied (Table 1).

3.4. *Production of methane from the effluent collected from DF*

During anaerobic digestion of the collected DF effluent, an increase in the organic loading rate from 6.0 to 10.5 g COD/L/day resulted in an increase in methane productivity (Table 2). Further increase in the organic loading rate to 15.4 g COD/L/day (1.0 day HRT) resulted in an increased methane production rate, i.e. 3.95 L/L/day, after 6 days of treatment time (data not shown). At a stable organic loading rate of 10.5 g COD/L/day (equivalent to 1.5 days HRT) a maximum methane production rate of 2.64 L/L/day (Table 2) was observed. The methane yield ranged from 0.28 to 0.26 L/g COD independent of the OLR and the methane content in biogas was about 60% (Table 2).

Stable operational conditions prevailed throughout the entire treatment period. The pH remained stable at around 7.50 for all applied OLRs. The effluent of the UASB reactor

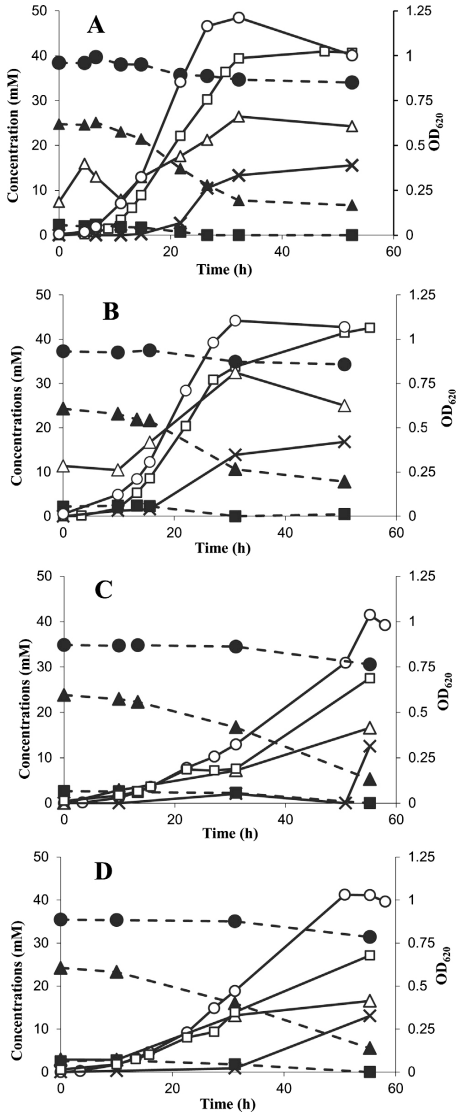

Fig. 2 – Batch fermentation profile of *C. saccharolyticus* cultures performed in closed serum flasks (Substrate, atmosphere in the headspace). WSH, N_2 (A), WSH, CH_4 (B), Pure sugars (glucose, xylose and arabinose), N_2 (C) and pure sugars, CH_4 (D). Glucose (●), xylose (▲), arabinose (■), OD_{620} (○), H_2 accumulation (□), lactate (✖) and Acetate (△). Each experiment is a representative of at least two independent replicates.

Table 1 – Results of the continuous fermentations of wheat straw hydrolysate by *C. saccharolyticus*.

Parameter	Results obtained at HRT (day) and at a sparging condition of:			
	0.83 (N_2) Case I	0.28 (N_2) Case II	0.83 (N_2 + CO_2) Case III	0.28 (N_2 + CO_2) Case IV
Biomass conc. (g/L)	1.25	1.07	0.47	0.54
(Q_{H_2})[b](L H2/L/day)	5.09	5.19	2.04	3.75
(q_{H_2})[c](L H2/g/day)	4.1	4.9	4.4	7.0
qsugar[d](g/g/day)[a]	5.3, 3.1, 0.3	8.4, 8, 0.8	3.7, 5.8, 0.8	7.5, 8.6, 0.8
Residual sugar[a](g/L)	0.9, 0.3, 0	3.9, 1.1, 0.05	5, 1.3, 0	5.4, 2.3, 0.2
Product yield (mol/mol)				
H_2	3.43	2.08	3.16	3.04
Acetate	1.69	1.07	1.75	1.66
Lactate	0.03	0.58	0.03	0.03
Ethanol	0.07	0.09	0	0.19
Conversion H2/total sugar (%)	88	46.3	33.4	19.2
Inhibitor reduction (%)				
HMF	32	5	16	20
Furfural	62	75	100	85
Osmolality[e]	0.23	0.21	0.25	0.25
Carbon recovery (%)	110	115	105	116
Redox recovery (%)	104	108	101	109

a Three values for three sugars, i.e. glucose, xylose and arabinose respectively.
b (Q_{H_2}), volumetric hydrogen productivity.
c (q_{H_2}), specific hydrogen productivity.
d q_{sugar}, specific sugar consumption rate.
e Osmolality was measured in Osmol/kgH_2O.

contained low concentrations of COD (<1 g/L) and volatile fatty acids (<0.1 g/L). Furthermore, the COD of the medium fed to the UASB reactor was reduced by approx. 95% after the treatment. Addition of modified anaerobic medium resulted in a need of a high reactor buffer capacity, which was reflected in the partial alkalinity that ranged from 5.4 to 5.8 g/L. The concentration of the buffer species NH_4^+–N, in the reactor varied from 0.66 to 0.74 g/L as a consequence of urea mineralization (Table 2).

Table 2 – Treatment of dark fermentation effluent in a UASB reactor.

Parameter	*HRT* (day)	
	2.5	1.5
Duration (days)	29	20
pH of influent	6.9	–
Influent COD	15.3	15.3
NH_4 – N (g/L)	0.12	0.12
OLR[a] (gCOD/L/day)	6.0 ± 0.5	10.5 ± 1.2
MPR[b] (l CH_4/L/day)	1.64 ± 0.12	2.64 ± 0.04
Methane yield (l CH_4/g COD)	0.28 ± 0.03	0.26 ± 0.04
Methane content (%)	60 ± 1	61 ± 4
pH of effluent	7.5	7.53
Effluent COD (g/l)	0.79 ± 0.05	0.78 ± 0.03
COD reduction (%)	95	94
Volatile fatty acids (g/l)	<0.01	0.06 ± 0.03
Partial alkalinity (g/l)	5.8 ± 0.2	5.4 ± 0.1
NH_4 – N (g/L)	0.74 ± 0.02	0.66 ± 0.11

a OLR, organic loading rate.
b MPR, methane production rate.

3.5. *Overall energy output*

On average, about 50% of the energy in wheat straw has been retrieved across all the scenarios of the *hythane* process. The energy output from DF was highest for *Case I* and lowest for *Case IV*. Although, the composition of effluent generated during different *Cases* of DF was different, due to the mixing of all the effluent together before its treatment, a scenario-specific energy output could not be determined for AD. Hence, a maximum energy output observed during AD was assumed to be true in all the scenarios of *hythane* (Table 3), which was significantly higher than the energy output from any of the DF *Cases* (Table 3). About 85% of the overall energy present in straw is contained in the sugars, of which 60% (average of all *hythane* scenarios, Table 3) has been successfully retrieved in the form of H_2 and CH_4 in the present *hythane* process.

4. Discussion

4.1. *Dark fermentation*

In this study, *C. saccharolyticus* was successfully cultured on WSH, provided that the concentration of WSH is less than 20% (v/v). *C. saccharolyticus* has been seen previously to grow efficiently on hydrolysates of wheat straw and Miscanthus, juices of sweet sorghum and sugar beet as well as on raw feedstocks, such as, maize leaves, *Silphium trifoliatum* leaves, potato peels, carrot pulp and paper sludge [34–39]. *C. saccharolyticus* has been observed to sustain growth in a medium containing up to 2 g/L of common growth inhibitors found in WSH, viz., 5-

Table 3 – Energy output in all scenarios compared with reference scenario. Values for energy contained in wheat straw (19.1 kJ/g) and in its sugar fraction (16.3 kJ/g) were obtained from Kaparaju et al. [3] and Nkemka et al. [27] respectively.

Scenario:	*Case I* + AD	*Case II* + AD	*Case III* + AD	*Case IV* + AD	*Case V*[c]
Energy output (kJ/g straw)					
H_2 Production (LCV[a])	2.3	0.8	0.9	0.6	–
CH_4 Production (LCV)[b]	8.6	8.6	8.6	8.6	11.6
Total	10.9	9.4	9.5	9.2	11.6
Energy yield (%)					
LCV Products/energy in straw	57	49	50	48	61
LCV Products/energy in sugars	67	58	58	56	71

a LCV, lower calorific values.
b Since the effluent collected from different *Cases* of DF was mixed before its treatment in AD, the energy output for the latter was assumed constant in all the scenarios in this study.
c A reference case scenario wherein WSH was directly fed to an AD reactor [27].

(hydroxymethyl)furfural and/or furfural [34]. However, the concentrations of these inhibitors in the WSH used in this study were far below 2 g/L [27]. On the other hand, the osmolality of the medium containing 20% WSH was found to be about 0.26 Osmol/kg of H_2O, which is well above the critical osmolality, i.e. 0.22 Osmol/kg of H_2O, reported for substantial growth inhibition in a growing culture of *C. saccharolyticus* [23]. Hence, the inability of *C. saccharolyticus* to initiate growth on higher concentrated WSH is related to its limited osmotolerance.

The results herein revealed that *C. saccharolyticus* is as unaffected by CH_4 as by N_2. To our knowledge no information is available in the literature about the ability of thermophiles like *C. saccharolyticus* to grow in the presence of CH_4. Performance on WSH (10% v/v) was slightly better than on artificial medium, which might be due to the presence of marginal amounts of soluble proteins and amino acids in WSH [8,9,27]. No obvious explanation could be found for the observed slight beneficiary effect of the presence of CH_4 compared to N_2 (Figs. 1(B) and 2). Nevertheless, it strongly suggests that sparging with upgraded CH_4 can be an appropriate alternative. However, to obtain purified CH_4, CO_2 should be removed from the flue gas of the AD reactor, which will incur significant additional costs. To reduce these costs, the DF reactor can be sparged with the non-upgraded flue gas of the AD reactor i.e. mixture of CH_4 and CO_2. In addition, *C. saccharolyticus* can sustain growth in non-sparging conditions in the reactor [22], which opens an opportunity to alleviate the costs of sparging. However, H_2 yields obtained in the absence of sparging are much lower due to formation of more undesirable by-products such as lactic acid, which is also not a preferred substrate for acetoclastic methanogenesis in AD [40,41]. Hence, absence of sparging in the DF reactor can affect both DF and AD. A thorough techno-economic evaluation of the entire process may conclude the best applicable alternative.

The maximum overall H_2 productivities observed in the *hythane* scenario (*Case I*, Table 1) is at least five times higher than the average H_2 productivity reported by Kongjan et al. [9]. Moreover, the productivities observed in all the *Cases* in this study are comparable to previously reported values for *C. saccharolyticus*, ranging from 2.3 to 9.7 L of H_2/L/day, the highest of which was achieved when hydrolysed potato steam peels were used as a substrate [14,34–38]. The observation of significantly lower H_2 yield in *Case II* may have been due to overflow metabolism, i.e. high glycolytic flux causing a metabolic shift at the pyruvate node to lactate formation. Overall, the combination of low biomass, volumetric H_2 productivity and sugar conversion efficiency of cultures sparged with N_2 + CO_2 clearly illustrate the dramatic effect of CO_2 in the sparging gas (*Case III and IV*, Table 1). A previous investigation on the effect of sparging with CO_2 in *C. saccharolyticus* cultures [23], revealed that the inherent formation of bicarbonate increased the osmotic potential to critical levels. As a consequence, extensive cell lysis occurs in the culture resulting in higher protein and DNA concentration in the culture broth [23]. Nevertheless, this nutrient-rich lysate might benefit the growth of the remaining cells, therefore displaying higher specific H_2 production rates observed in cultures sparged with CO_2 (*Case III and IV*, Table 1). Alternatively, the observation of CO_2 stimulating growth of *C. saccharolyticus* on xylose [42] might have improved specific H_2 productivity in *Case III* and *IV*.

None of the *Cases* studied showed complete consumption of sugars which could indicate a limitation of an essential nutrient. It can be argued that it might be sulphur. Firstly, phosphoric acid (H_3PO_4), instead of sulphuric acid (H_2SO_4), was used in the mild acid pretreatment of wheat straw used in this study, thus eliminating a potential sulphur source from the medium [27]. Secondly, the influents of all DF cases were supplemented with yeast extract as the only sulphur source. With a minimal concentration of 1 g/L it may not have provided adequate amounts of sulfur. Finally, wheat straw itself contains very negligible amounts of sulfur [43]. However, further experiments are needed to explore this hypothesis as they were out of the scope of this study.

The higher carbon and electron (redox) recovery observed in all the cases may have been due to traces of non-hydrolyzed disaccharides and/or oligosaccharides in WSH. This also may have resulted in a possible overestimation of H_2 yields in the respective cases.

4.2. *Anaerobic digestion of the effluent collected from DF*

The maximum methane production rate obtained during anaerobic digestion of the DF effluent collected from a H_2 producing CSTR during this study is significantly higher than a previously reported value (2.1 L CH_4/L/day) in a similar study where DF effluent was collected from a H_2 producing UASB

reactor [9]. This might be related to the differences in composition of DF effluent, as: i) the DF effluent collected during this study contained mainly acetate whereas, its counterpart in the previous study contained significant amounts of butyrate, propionate and ethanol, along with acetate [9], and ii) acetoclastic methanogens take acetate as a substrate and rely on acetogens for the conversion of butyrate, propionate and ethanol to acetate [40,41]. In another study [27], WSH was directly fed to a methanogenic UASB reactor at an OLR of 10.2 g COD/L/day producing methane at a production rate (2.7 L CH_4/L/day) comparable with the one reported in the present study.

So far, sustained organic loading rates up to 15 g COD/L/day have been reported in the treatment of DF effluents in a UASB reactor [3,9,44,45]. However, applications of OLRs higher than 15 g COD/L/day were observed to result in accumulation of volatile fatty acids, low COD reductions and low CH_4 yields. In addition, very high OLRs generate vigorous gas production rates, thus inflicting instability to the granular bed and eventually leading to process failure [45]. Due to a decrease in methane yield and slight increase in VFA accumulation at higher OLR (10.2 g COD/L/day, Table 2) further increase in OLR was abandoned in this study.

A stable pH within the range of 7–8 has been reported as optimum for acetoclastic methanogenesis [9]. Consumption of VFA during AD may have contributed to a pH increase to a suitable range.

Granular anaerobic sludge is known to be more protective for methanogens against inhibitory compounds than liquid granular sludge [46]. This could be a reason why batch tests of AD using liquid anaerobic sludge resulted in lower CH_4 yields on DF effluent (~0.22 L CH_4/g COD) than obtained from effluent treated in the UASB reactor with granular anaerobic sludge (Table 2).

4.3. *Overall energy output and the potential of the process*

The overall energy yield obtained during this study (average of all *hythane* scenarios), i.e. approximately 2010 kJ/L of WSH, was about four times higher than the stable overall energy yield reported earlier for a similar study (440 kJ/L of WSH, estimated from Ref. [9]). Thus, in comparison, this study reports a very efficient process with respect to overall energy output. However, in the study performed by Kongjan et al. [9], the total sugar concentration in the culture medium was about twice lower than in this study, which resulted in comparatively lower H_2 and CH_4 yields per litre of WSH and consequently a lower energy yield.

Another study on biohydrogen production from WSH reports an energy yield of 0.96 kJ/g of wheat straw (estimated from Refs. [3,8]) which is two-folds lower than the energy yields obtained in *Case I* (Table 3) of the DF phase studied herein. In the present study, the overall conversion efficiency for a *hythane* process i.e. 60% could not match the high conversion efficiency i.e. 71% obtained in a study pertaining to production of biogas using WSH (Table 3 [27],). However, the former will be advantageous, if the aim is to produce *hythane*.

About 85% of the energy in wheat straw can be retrieved in the form of soluble sugars (Table 3). Although, reasonably high substrate conversion efficiencies can be achieved during DF and AD using the soluble sugars in WSH; the possible losses of sugars during the extensive pre-treatment process can result in much lower overall energy yields (Tables 1–3). Hence, an efficient pre-treatment process is of paramount importance for any *hythane*-like process.

In the current study, the AD expending about five-folds more process time than DF (1.5 days for AD and 0.28 days for DF), will consequently require reactors with five-folds more volumetric capacity than DF. Reactors with higher volumetric capacity will incur higher capital and operational costs. This can be conveniently avoided simply by operating DF reactors at high HRT (preferably similar to that of AD), which may also aid in achieving higher conversion during DF (Table 1 and 3).

Overall, the process offers a number of benefits with respect to convenience in operation and cost, i) a thermophilic DF process offers less risk of contamination by H_2-oxidising methanogens in the DF reactor [47], ii) the contaminants can also be kept out of the DF reactor by operating it at relatively higher growth rate [8] and iii) the process can successfully retrieve about 57% of the energy present in wheat straw. More technical details of the process and possible ways of cost reduction have been extensively discussed elsewhere [48].

5. Conclusions

C. saccharolyticus can efficiently produce H_2 from sugars in WSH. The residual sugars and acids produced can subsequently be converted to CH_4 in a methanogenic UASB reactor. The two-step process gives reasonable conversion efficiencies (about 67% of energy in the sugar fraction of wheat straw), but there remains room for further improvement. Moreover, the performance of *C. saccharolyticus* is not affected by CH_4 allowing application of this gas for sparging the hydrogenogenic reactor. However, a further extensive techno-economic evaluation is required to determine the best DF set up out of the following scenarios: i) sparging with upgraded CH_4, ii) sparging with the non-upgraded flue gas from the AD reactor, or iii) no sparging. An optimized and economically feasible version of this process can potentially complement a bio-refinery, wherein, along with bio-energy other value-added products are also produced from any unutilized parts of renewable agricultural biomass. This study paves a way for further exploration to determine whether a biological *hythane* process can be a viable alternative for the conversion of lignocellulosic biomass.

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Paper III

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Evaluation of assimilatory sulphur metabolism in *Caldicellulosiruptor saccharolyticus*

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HIGHLIGHTS

- *C. saccharolyticus* can assimilate sulphate and grow in absence of a reducing agent.
- Majority of *Caldicellulosiruptor* species possess genes for sulphate assimilation.
- *C. saccharolyticus* tolerates high P_{H_2} regardless of sulphur source used.
- Sulphate can replace cysteine in the cultivation medium of *C. saccharolyticus*.

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ABSTRACT

Caldicellulosiruptor saccharolyticus has gained reputation as being among the best microorganisms to produce H_2 due to possession of various appropriate features. The quest to develop an inexpensive cultivation medium led to determine a possible replacement of the expensive component cysteine, i.e. sulphate. *C. saccharolyticus* assimilated sulphate successfully in absence of a reducing agent without releasing hydrogen sulphide. A complete set of genes coding for enzymes required for sulphate assimilation were found in the majority of *Caldicellulosiruptor* species including *C. saccharolyticus*. *C. saccharolyticus* displayed indifferent physiological behaviour to source of sulphur when grown under favourable conditions in continuous cultures. Increasing the usual concentration of sulphur in the feed medium increased substrate conversion. Choice of sulphur source did not affect the tolerance of *C. saccharolyticus* to high partial pressures of H_2. Thus, sulphate can be a principle sulphur source in an economically viable and more sustainable biohydrogen process using *C. saccharolyticus*.

1. Background

The growing demand for merchant hydrogen supplies has fuelled research interests for the development of commercially viable biological hydrogen production processes. Thermophilic hydrogen production from lignocellulosic biomass is one of the most promising processes of the near future. *Caldicellulosiruptor saccharolyticus* is an extremely thermophilic obligate anaerobe, possessing a natural ability of producing hydrogen from a wide range of mono-, di- and oligosaccharides (Bielen et al., 2013a,b). *C. saccharolyticus* is one of the best, yet not ideal, amongst naturally occurring hydrogen-producing organisms for a number of metabolic features it possesses (Pawar and van Niel, 2013).

The enzymes involved in hydrogen metabolism, i.e. [NiFe]-, and [FeFe]-hydrogenases, Pyruvate:ferredoxin oxidoreducatases (PFOR) and ferredoxin are all containing Fe–S clusters (TranQui and Jesior, 1995; Cavazza et al., 2006; Kim and Kim, 2011) making sulphur (S) more than an essential element for optimal growth of *C. saccharolyticus*. In addition, obligate anaerobes are known to require a reducing agent in the medium for creating an environment with lower minimum redox potential. Interestingly, the majority of applied reducing agents contain the reduced form of sulphur, such as cysteine, di-thio-threitol and sodium sulphide, contain reduced form of sulphur.

Historically, in the studies with *C. saccharolyticus*, cysteine has primarily been used as a reducing agent (Kadar et al., 2004; de Vrije et al., 2007; Ivanova et al., 2009; Mars et al., 2010; Willquist et al., 2011), but in fact it was also the only significant source of sulphur in the medium (Zeidan, 2011). Indeed, in a previously reported study, inclusion of yeast extract (1 g/L) instead of cysteine in the feed medium led to very low consumption of sugars, indicating limitation of sulphur in the medium (Pawar et al., 2013).

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In general, cysteine is widely used for various reasons – (i) as an organic source of sulphur, (ii) as a nutritional source, (iii) as a reducing agent by reducing reactive oxygen species in the medium and/or, (iv) as a chelating agent for iron (II/III) (Dognin and Crichton, 1975; Verduyn et al., 1992). However, besides yeast extract cysteine is the most expensive component in the cultivation medium used for *C. saccharolyticus* (Ljunggren and Zacchi, 2010). In addition, upon mineralisation cysteine can be a source of H_2S gas (Morra and Dick, 1991), which will cause irreversible damage to fuel cells and other equipment (Mohtadi et al., 2003; Shi et al., 2007). Hence, finding a cheaper and more sustainable alternative to cysteine will contribute to more cost-effective H_2 production by *C. saccharolyticus.* In this study, we aim to evaluate sulphate salts as an alternative to cysteine through studying its effect on the metabolism of *C. saccharolyticus* under different growth conditions.

2. Methods

2.1. Microorganism and its maintenance

C. saccharolyticus DSM 8903 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Routine subcultures and maintenance were conducted in 250-mL serum bottles containing 50-mL of a modified DSM 640 medium (Willquist et al., 2009) unless stated otherwise. Anoxic solutions of glucose, cysteine·HCl, and $MgSO_4{\cdot}6H_2O$ were autoclaved separately and added to the sterile medium at the required concentration. A 1000× concentrated vitamins solution was prepared as described previously (Zeidan and van Niel, 2010) and used in the growth medium at 1× concentration as a replacement to yeast extract.

Chelating agents prevent precipitation of minerals in the medium by chelating the divalent ions, and for that reason have been widely used medium formulations, such as Verduyn medium (uses EDTA, (Verduyn et al., 1992)) and Wolfe's mineral medium (uses Nitrilotriacetic acid, (Wolin et al., 1963)). Most of the previous studies on *C. saccharolyticus* were performed based on DSM 640 medium, but have omitted chelating agents in the trace elements solution – SL-10 (van Niel et al., 2003; Zeidan and van Niel, 2010; Bielen et al., 2013a,b; Talluri et al., 2013). Yet, some studies on *C. saccharolyticus* have used other media formulations containing EDTA (Isern et al., 2013). In the present study, severe precipitation was observed in the medium containing SL-10 when the $MgSO_4{\cdot}6H_2O$ concentration was increased leading to experimental failures (data not shown). Therefore, for all the experiments a 1000× concentrated trace element solution (modified SL-10) was prepared as described previously (Zeidan and van Niel, 2010), except for the addition of 1.5 g/L of Nitrilotriacetic acid.

2.2. Fermentation setup and culture medium

Cultivations were performed either in batch or continuous mode wherein the elemental concentration of sulphur in its culture medium was varied as per experimental condition. All the experiments were conducted in a jacketed, 3-L bioreactor equipped with an ADI 1025 Bio-Console and an ADI 1010 Bio-Controller (Applikon, Schiedam, The Netherlands) at a working volume of 1L, either in batch or continuous mode. The pH was maintained at 6.5 ± 0.1 at 70 °C by automatic titration with 4 M NaOH. The temperature was thermostatically kept at 70 ± 1 °C. A condenser with 5 °C cooling water was fitted to the bioreactor's headplate. During batch cultivations, culture samples were collected at different time intervals for monitoring growth and the culture supernatant was collected for analysis of glucose, acetic acid, lactic acid, propionic acid and ethanol. Gas samples were collected from reactor's headspace to analyze levels of H_2 and CO_2. Samples for NAD(H), NADP(H), ATP and PPi measurements were collected once during early exponential phase and later in the stationary phase. Similarly, for continuous cultures, samples were collected at steady state. Batch cultures were performed in two independent biological replicates, whereas, for continuous cultures steady states were obtained in technical duplicates.

To study the effect of different sulphur sources on the growth and H_2 tolerance of *C. saccharolyticus*, the batch cultivations were performed using the medium containing either cysteine. HCl (*Case* I) or $MgSO_4{\cdot}7H_2O$ (*Case* II). The reactors were autoclaved containing a defined cultivation medium (DM, per L): KH_2PO_4 0.75 g; $K_2HPO_4{\cdot}2H_2O$ 1.5 g; NH_4Cl 0.9; resazurin 1 mg, 1000× modified SL-10 1 mL. Solutions of glucose, 10 g/L, and cysteine·HCl, 0.64 g/L (*Case* I, [S] = 0.117 g/L) or $MgSO_4{\cdot}6H_2O$, 0.9 g/L (*Case* II, [S] = 0.117 g/L) were autoclaved and added separately prior to inoculation. $MgCl_2{\cdot}7H_2O$, 0.4 g/L, was added to the cultures containing cysteine·HCl (*Case* I). 1 mL of filter-sterilized 1000× vitamins solution was added to the reactor after the medium had turned completely anaerobic to avoid possible degradation. Cells of *C. saccharolyticus* were sub-cultured twice in serum bottles with the same medium as that of the respective batch and the third subculture was used as inoculum. For *Case* II, instead of adding a reducing agent to the medium, the subcultures and reactors were sparged with N_2, prior to inoculation, for much longer duration compared to *Case* I. For both *Cases* I and II, reactors were not sparged during the cultivation and the pressure was maintained at atmospheric level by allowing accumulation of H_2 and CO_2 in the headspace and further collection in an inverted graduated cylinder filled with saturated solution of $NaHCO_3$.

For continuous cultivations, the bioreactor was started to be fed with fresh medium at the end of the logarithmic growth phase of the batch culture. The basic composition of the feed media were (g/L): KH_2PO_4 0.75; $K_2HPO_4{\cdot}2H_2O$ 1.5; Resazurin 1 mg/L, 1000× 'modified SL-10' 1 (mL/L) and 1000× vitamins solution (1 mL/L). The composition of the feed was varied regarding the source and concentration of sulphur (Table 1). Since, cysteine·HCl can also provide nitrogen, Feed C and D were provided with additional amounts of NH_4Cl (Table 1). Similarly, since, $MgSO_4{\cdot}6H_2O$ can provide Mg^{2+}, only the feeds containing cysteine·HCl (Feed A and E) were supplemented with $MgCl_2{\cdot}7H_2O$ (Table 1). Glucose was used as a primary substrate in all continuous experiments at an initial concentration of 10 g/L. Steady states were assessed after at least 5 volume changes based on the criteria of constant H_2 and CO_2 production rates and constant biomass concentration.

2.3. Analytical methods

Headspace samples were analysed for CO_2 and H_2 by gas chromatography, using a dual channel Micro-GC (CP-4900; Varian, Micro gas chromatography, Middelburg, The Netherlands), as previously described (Zeidan and van Niel, 2010). The results were

Table 1
Variations in the composition of the different feed media (g/L) used in continuous cultures of *C. saccharolyticus*. S_1, $MgSO_4{\cdot}6H_2O$; S_2, Na_2SO_4; S_3, Cysteine·HCl; N, NH_4Cl; Mg, $MgCl_2{\cdot}7H_2O$. Feed A and B contained much lower [S] compared to that of Feed C, D and E.

	S_1	S_2	S_3	N	Mg	[S] g/L
Feed A	0	0	0.35	0.9	0.4	0.064
Feed B	0.5	0	0	0.9	0	0.065
Feed C	0.62	0.25	0	1.12	0	0.137
Feed D	0.62	0	0.3	1.04	0	0.136
Feed E	0	0	0.75	0.9	0.4	0.137

analysed with a Galaxie Chromatography Workstation (v.1.9.3.2). The optical density of the culture was measured at 620 nm (OD_{620}) using a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). CDW was determined by filtration as previously described (Willquist and van Niel, 2010). Glucose, acetate, lactate, propionate and ethanol were analyzed by HPLC (Waters, Milford, MA, USA) on an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA) at 45 °C, with 5 mM H_2SO_4 (0.6 ml min^{-1}) as mobile phase. The column was equipped with a refractive index detector (RID-6A; Shimadzu, Kyoto, Japan).

2.4. Determination of intracellular levels of ATP and PPi

1 mL of culture samples were collected in screw-cap micro-centrifuge tubes containing ice-cold chloroform and immediately frozen into liquid nitrogen. Samples were stored at −80 °C until further analysis. Intracellular concentrations of ATP were measured with an ATP Bioluminescence assay kit HSII (Roche Molecular Biochemicals, Germany) as described earlier (Willquist et al., 2011). Intracellular concentrations of PPi were measured as previously described (Willquist et al., 2011), except for the following modification. Instead of subtracting the background signal from ATP present in the samples, ATP was denatured by incubating the samples at 70 °C for about 4 h before performing assays for PPi measurement. A number of control assays were performed to confirm the stability of PPi and denaturation of ATP under the incubating conditions.

2.5. Measurements of NAD(P)(H)

The intracellular concentrations of NADH and NAD^+ were determined by a cyclic assay as described earlier (Willquist et al., 2011). Similarly, levels of NADP(H) were determined except for the following modifications – a) NAD^+ *alcohol dehydrogenase* (EC 1.1.1.1, ADH) was replaced by $NADP^+$ specific ADH (EC 1.1.1.2) and b) NADPH standards were used instead of NADH.

2.6. Detection of hydrogen sulphide production

Hydrogen sulphide (H_2S) detection strips (Sigma–Aldrich, Germany) were used to monitor production of H_2S by

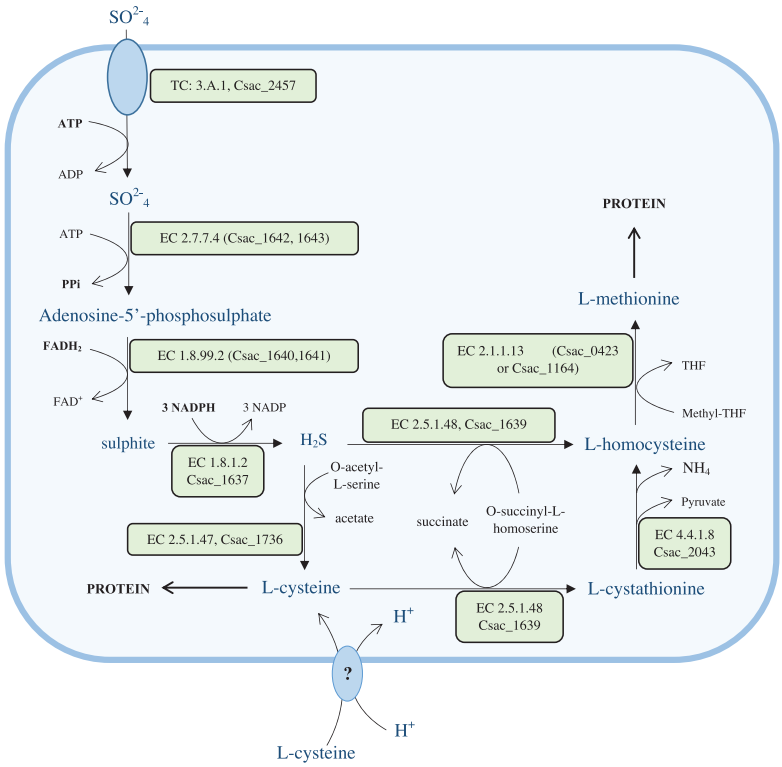

Fig. 1. Assimilatory sulphur metabolism in *C. saccharolyticus* (re-annotated after Zeidan, 2011). TC: 3.A.1 – ATP-binding cassette superfamily, EC 2.7.7.4 – sulfate adenylyltransferase, 1.8.99.2 – adenylylsulfate reductase, EC 1.8.1.2 – sulfite reductase, EC 2.5.1.47 – cysteine synthase, 2.5.1.48 – O-succinylhomoserine succinate-lyase, EC 4.4.1.8 – cystathionine-β-lyase and EC 2.1.1.13 – methionine synthase. Although, no candidate gene was found, uptake of L-cysteine is assumed to be via proton symporter (Zeidan, 2011).

C. saccharolyticus by hanging the strips through the rubber caps of serum flasks containing DM with either $MgSO_4 \cdot 6H_2O$ or cysteine incubated with or without cells of *C. saccharolyticus*.

2.7. Re-annotation of sulphur assimilation pathway in Caldicellulosiruptor species

Sulphur assimilation pathways in *C. saccharolyticus* were reconstructed previously (Zeidan, 2011), however, the proposed pathways were re-annotated during this study. All the information regarding genome sequences and corresponding annotations were retrieved from the Integrated Microbial Genomes (IMG, https://img.jgi.doe.gov/cgi-bin/w/main.cgi). The candidate genes within genomes of all the candidates in genus *Caldicellulosiruptor*, coding for enzymes taking part in assimilatory sulphur metabolism were identified based on enzyme commission (EC) numbers. Once a candidate gene was identified, corresponding orthologs present in other *Caldicellulosiruptor* species were found using the homolog-search tools available on the IMG website.

2.8. Calculations

H_2 productivity ($mM\ h^{-1}$) and cumulative H_2 formation (CHF, mM) were calculated in two different ways depending on the experimental design. All calculations were based on the ideal gas law and the H_2 and CO_2 concentrations in the headspace. For continuous cultures, the calculations were based on the flow rate of the influent N_2 gas and the percentages of H_2 and CO_2 in the effluent gas, as no other gases were detected. For batch experiments without sparging the flow rate of the effluent gas was measured by the 'liquid displacement method' using a $NaHCO_3$-saturated solution to avoid any further CO_2 solubilisation. Thus, H_2 productivity and CHF were calculated based on hydrogen concentration in the effluent gas and the flow rate of the effluent gas. Product yields were calculated by determining moles of products formed per mole of glucose consumed. Biomass yield was calculated as moles of biomass formed per mole of glucose consumed. Carbon and redox balances were calculated as described previously (de Vrije et al., 2007). The catabolite reduction charge and anabolic reduction charge are defined as $NADH/(NADH + NAD^+)$ and $NADPH/(NADPH + NADP^+)$, respectively.

3. Results and discussion

3.1. Sulphur assimilation in Caldicellulosiruptor species

As described in previous studies, all *Caldicellulosiruptor* species can assimilate sulphur through cysteine. However, only a few of them, including *C. saccharolyticus*, can use sulphate as well. Among all species in the genus, so far, only *Caldicellulosiruptor bescii* has been reported to use sulphate (Kridelbaugh et al., 2013). However, during that study, the growth experiments were performed in unoptimised conditions of pH and temperature. Moreover, the effects of sulphate assimilation on the physiology of *C. bescii* were not studied in detail (Kridelbaugh et al., 2013). Another study reports the use of Na_2S as a replacement to cysteine for cultivation of *C. bescii* (Farkas et al., 2013), albeit disregarding the hazardous nature of this reduced compound.

For the reannotation of the assimilatory sulphur metabolic pathways in *C. saccharolyticus* two independent sulphur assimilation systems were studied, i.e. one for the uptake of sulphate and other for cysteine (Fig. 1). Among all the *Caldicellulosiruptor* species available, genes coding for sulphate-uptake and assimilation were found in the majority of the genomes except for those of *Caldicellulosiruptor owensensis*, *Caldicellulosiruptor lactoaceticus* and

Table 2
Assimilatory sulphur metabolism in genus Caldicellulosiruptor.

Organism	Genes coding for enzymes involved in sulfur assimilation pathway							
	EC2.7.7.4	EC 1.8.99.2	EC 1.8.1.2	EC 2.5.1.48	EC 2.1.1.13	EC 2.5.1.47	EC 4.4.1.8	?
C. saccharlyticus	Csac_1642, -1643	Csac_1640,-1641	Csac_1637[b]	Csac_1639	Csac_0423[a], -1164	Csac_1736	Csac_2043[a]	Csac_2457[f]
C. owensensis	?	?	?	?	Calow_2158[d]	Calow_1359	Calow_1275[d]	?
C. kristjanssonii	Calkr_1177	Calkr_1174	CaldiKris_00011570	Calkr_1175[e]	Calkr_0114[d]	Calkr_1148, -1627	Calkr_1481[e], CaldiKris_00001140	Calkr_1789[f], CaldiKris_00004040
C. bescii	Athe_1194	Athe_1191	Athe_1188	Athe_1190[c]	Athe_2582	Athe_1165,-1615	Athe_1496[e]	?
C. hydrothermalis	Calhy_1539	Calhy_1542[a]	Calhy_1535,36,37	Calhy_1543[c]	Calhy_0262[d]	Calhy_1137,-1569	Calhy_1240[e]	Calhy_2134[f]
C. kronotskyensis	Calkro_1504	Calkro_1507[a]	Calkro_1510	Calkro_1508[c]	Calkro_0279[d]	Calkro_1110,-1531	Calkro_1214[e]	Calkro_2114[f]
C. lactoaceticus	?	?	?	?	Calla_2222[d]	Calla_1023	Calla_0870[e]	Calla_1187[f]
C. obsidiansis	?	?	?	?	COB47_2197[d]	COB47_0967	COB47_1058[e]	?

[a] Amino acid sequence homology with Csach_1640 > 90%.
[b] Amino acid sequence homology with CaldiKris_0001570 > 90%.
[c] Amino acid sequence homology with Csach_1639 ⩾ 90%.
[d] Amino acid sequence homology with Athe_2582 > 95%.
[e] Amino acid sequence homology with CaldiKris_00001140 > 80%.
[f] Amino acid sequence homology with CaldiKris_00004040 > 80%.

Table 3
Results obtained from sulfur-limited chemostats performed under various conditions. Composition of various 'Feed' is given in Table 1.

Parameter	Results obtained at different D (h^{-1}) using different sources of sulfur in the culture medium						
	Feed A @ 0.1 *D*	Feed A @ 0.2 *D*	Feed B @ 0.1 *D*	Feed B @ 0.2 *D*	Feed C @ 0.2 *D*	Feed D @ 0.2 *D*	Feed E @ 0.2 *D*
Biomass conc. (g/L)	0.41 ± 0.01	0.26 ± 0.01	0.36 ± 0.01	Washout	0.44 ± 0.02	0.44 ± 0.07	0.45 ± 0.11
$q_{glucose}$ (mmol/g/h)	3.4 ± 0.3	3.6 ± 0.3	4.8 ± 0.1	–	4.44 ± 0.07	5.4 ± 0.64	5.2 ± 1.1
$q_{acetate}$(mmol/g/h)	5.4 ± 0.2	6.28 ± 0.06	7.2 ± 0.2	–	6.5 ± 0.3	7.6 ± 1.2	8.5 ± 1.2
q_{H_2} (mmol/g/h)	12.6 ± 0.4	11.87 ± 0.09	16.22 ± 0.08	–	16.5 ± 0.6	19.3 ± 2.9	21.9 ± 5.2
Product yield (mol/mol)							
H_2	3.6 ± 0.2	2.9 ± 0.2	3.37 ± 0.07	–	3.7 ± 0.2	3.45 ± 0.04	3.50 ± 0.06
Acetate	1.61 ± 0.08	1.64 ± 0.02	1.49 ± 0.02	–	1.46 ± 0.09	1.47 ± 0.05	1.61 ± 0.08
Biomass	1.2 ± 0.2	2.3 ± 0.2	0.85 ± 0.03	–	1.83 ± 0.03	1.41 ± 0.09	1.64 ± 0.35
Ethanol	0	0.05 ± 0.04	0.15 ± 0.02		0.09 ± 0.02	0.06 ± 0.01	0.08 ± 0.01
Lactate	0.03 ± 0.02	0	0.07 ± 0	–	0	0	0
Redox potential (mV)	−474 ± 1	−437 ± 0	−568 ± 0	–	−570 ± 3.5	−501 ± 8	−511 ± 20
Carbon recovery (%)	103 ± 6	107 ± 6	102 ± 1	–	113 ± 7	97 ± 1	99 ± 3
Redox recovery (%)	104 ± 6	115 ± 7	100 ± 1	–	114 ± 7	100 ± 1	99 ± 3

Caldicellulosiruptor obsidiansis (Table 2). In *C. saccharolyticus*, sulphate is taken up by an *ABC-type sulphate transporter* at the expense of an ATP. Further, sulphate is primed with an adenylyl group by *sulphate adenylyltransferase* at the expense of another ATP, but yielding 1 mol of PPi. Sulphate is reduced in subsequent steps by, first using 1 mol of $FADH_2$ catalysed by *adenylsulphate reductase* to give sulphite and then by using 3 mol of NADPH to produce a mole of H_2S mediated by *sulphite reductase*. H_2S is readily assimilated into either cysteine or methionine catalysed by various *synthases* and/or *lyases*. No specific candidate gene(s) encoding for L-cysteine transporters were found across all *Caldicellulosiruptor* species studied (Table 2). Nevertheless, genes encoding for L-cysteine assimilation and conversion to L-methionine were observed in all of them (Table 2). Similar to a number of non-enteric bacteria, *C. saccharolyticus* possesses an adenosine-5′-phosphosulphate-dependent mechanism of sulphate assimilation, which does not involve the formation of 3′-phosphoadenosine-5′-phosphosulfate (Kopriva et al., 2002). Assimilation of sulphate incurs relatively major cost to the growing cells by expending 2 mol of ATP, a mole of $FADH_2$ and 3 mol of NADPH per mole of sulphate taken up. In the process, however, a mole of PPi is also produced, which reduces the energy expenses somewhat as it is an energy carrier in *C. saccharolyticus* (Bielen et al., 2010). In contrast, assimilation of cysteine does not incur any energy expenditure.

To evaluate if this excess energy expenditure during sulphate assimilation has any effect on *C. saccharolyticus*, continuous culture studies were performed using a chemically defined medium differing with respect to the source of sulphur and its concentration (Table 1). The chemostats fed with the medium containing low concentrations of sulphur were operated at two different dilution rates (D), i.e. at 0.1 and 0.2 h^{-1} (Table 1 and 3). Higher sulphur concentrations were only applied at 0.2 h^{-1}. Of the cultures grown at 0.1 h^{-1} with low sulphur concentration (Feed A and B, Table 3), the biomass concentration and biomass yield were significantly higher with cysteine than with sulphate. In contrast, with sulphate, higher specific glucose consumption, specific acetate and H_2 productivity were observed compared to cysteine. For cultures fed with 'Feed A' (Table 3), increase in D decreased the biomass concentration and increased the redox potential. Of all cultures at D = 0.2 h^{-1}, with low cysteine the hydrogen yield was the lowest, whereas the biomass yield was significantly highest. Although among the cultures growing on low sulphur concentration, only the one growing on sulphate (Feed B) suffered wash-out at higher growth rate (0.2 h^{-1}), the unusual high biomass yield obtained in cysteine-grown culture (Feed A) might indicate that close to wash-out conditions the culture invested relatively more in biomass formation. As the sulphur concentration was increased the cultures behaved as usual by diverting most of the substrate to H_2 formation. The wash-out at 0.2 h^{-1} for sulphate-grown cultures can be attributed to the absence of reducing agent in the medium, and the low biomass concentration further failed to maintain a viable redox in the culture broth.

Interestingly, the redox potential in the cultivations containing reducing agent (Feed A, D, and E, Table 3) was higher than its counterpart lacking any reducing agent (Feed B and C, Table 3). It can be hypothesized that continuous uptake of the reducing agent cysteine due to its function as sulphur source, increased the redox potential in extracellular milieu.

At 0.2 h^{-1}, at low sulphur concentration no steady state could be obtained for the culture using sulphate as the culture washed out indicated by the observed increase in redox potential to positive values (data not shown) eventually rendering the culture non-viable (Feed B, Table 3). Regardless of the sulphur concentration in the feed, lower extracellular redox potentials were observed in the cultures with sulphate compared to the ones with cysteine.

In chemostats operated at 0.2 h^{-1} and fed with higher sulphur concentrations (Feed C, D and E, Table 3), no significant differences were observed in biomass concentration, specific glucose consumption rate, specific productivities for acetate and H_2, or the H_2 and acetate yields, regardless of the S-source. Under the same conditions, the significantly higher biomass yield was obtained in the culture grown on sulphate (Feed C, Table 3) compared to the corresponding culture grown on the combination of sulphate and cysteine (Feed D, Table 3). Overall, regardless of the sulphur source, increasing the sulphur concentration from 0.065 to 0.137 g/L caused an increase in biomass and product concentrations indicating sulphur limitation at the lower concentration.

The values for specific glucose consumption rate ($q_{Glucose}$) and H_2 production rate (q_{H_2}) were lower if not similar to the results obtained in carbon-limited cultures fed with much less glucose (de Vrije et al., 2007; Willquist et al., 2011). C-limited cultures from previous studies, report a steady increase in the $q_{Glucose}$ and q_{H_2} concomitant to increase in the growth rate (de Vrije et al., 2007), which is in agreement with the S-limited cultures in the present study, albeit only for cysteine-grown cultures (Table 3). Overall, under favourable physiological conditions, both C- and S-limited cultures displayed similar flux distributions at the pyruvate node ((de Vrije et al., 2007; Willquist et al., 2011; Pawar et al., 2013), Table 3). All the carbon and electrons present in the carbon source were accounted for in the products obtained in all the cultures performed during the study (Table 3).

Since sulphate assimilation by *C. saccharolyticus* involves production of H_2S as an intermediate before being implemented into S-containing amino acids, experiments were performed to detect release of H_2S. The H_2S detection strips remained colourless in *C. saccharolyticus*' cultures grown in a defined medium containing

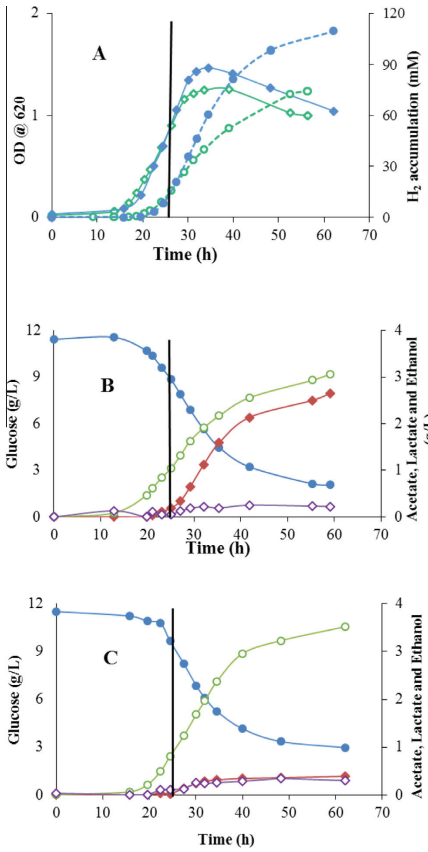

Fig. 2. Growth and product formation by *C. saccharolyticus* in pH-controlled batch fermentations without N_2 sparging containing different sulphur source: (A) with sulphate (open symbols, green) and with cysteine (filled symbols, blue) growth (diamonds) and P_{H_2} (circles). Product formation in sulphate-grown culture (B) and cysteine-grown culture (C), glucose (filled circle, blue), acetate (open circle, green), lactate (filled diamond, red) and ethanol (open diamond, purple). The vertical line represents the time point when the reactors became saturated with P_{H_2}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sulphate without cysteine or yeast extract, implicated that they did not release any H_2S into the headspace of the serum flasks. In contrast, the strips in the flasks containing cysteine and/or yeast extract, regardless of the presence of *C. saccharolyticus*, turned black indicating the release of H_2S to the headspace.

3.2. Growth and fermentation profile under high P_{H_2} with two different sources of sulphur in batch cultures

C. saccharolyticus is able to withstand P_{H_2} up to saturation levels (Willquist et al., 2011), which implies it would behave well under non-sparging conditions. This would make the hydrogen production process more cost-effective as it simplifies gas-upgrading (Ljunggren and Zacchi, 2010). Hence, the effect of high P_{H_2} on growth and H_2 production of *C. saccharolyticus* as a function of the sulphur source was evaluated. For both sulphur sources, i.e. sulphate (*Case* I) and cysteine (*Case* II), the headspace of the reactors reached saturation level, i.e. approx. 67% H_2 in headspace, with respect to the partial hydrogen pressure (P_{H_2}) within approximately 26 h and remained saturated until the end of the experiment (Fig. 2A). Even so, growth of *C. saccharolyticus*, glucose consumption, and production of acetate, lactate, ethanol and H_2 continued for hours until H_2 accumulation finally ceased (Fig. 2A–C). However, after reaching the 'P_{H_2} saturation' level, significant differences in the product accumulation were observed between *Cases* I and II. The maximum biomass, acetate and hydrogen concentrations were significantly lower in *Case* I compared to that of *Case* II. Although, for both *Cases* the beginning of 'P_{H_2} saturation' phase coincided with initiation of lactate and ethanol formation, overall lactate accumulation was significantly higher in *Case* I compared to its counterpart (Fig. 2B and C). Interestingly, no significant difference was observed in the final concentration of ethanol between *Cases* I and II (Fig. 2B and C). Sulphate can be considered as an oxidized compound as its standard potential is positive (0.20 V), whereas that of cysteine is negative (−0.34 V).Yet, assimilation of sulphate did not affect the tolerance of *C. saccharolyticus* to high P_{H_2}. Consistent with previous findings (Willquist et al., 2011), cultures showed higher propensity to produce lactate and ethanol soon after reaching saturation levels of P_{H_2}. In saturated P_{H_2} conditions, NADH-dependent hydrogen production becomes thermodynamically impossible (Willquist et al., 2011), shifting catabolism towards lactate and/or ethanol production to regenerate NAD^+. However, the ratio of reduced to oxidised organic products (lactate/acetate) was higher in the presence of sulphate than with cysteine, which is contradictory to the findings in a similar study with *Clostridium thermocellum* (Kridelbaugh et al., 2013). Moreover, assimilation of sulphate requires more reducing equivalents. Owing to the following characteristics of *C. saccharolyticus*: (i) absence of $NADP^+$ *transhydrogenase* and presence of *isocitrate dehydrogenase* (ICDH) as the only enzyme capable of producing NADPH (Zeidan, 2011), and (ii) lower acetate yield in cultures using sulphate (Fig. 2B); it can be hypothesised that – with increasing biomass, higher amounts of NAD^+ were needed in cultures growing on sulphate to generate $NADP^+$, which were immediately reduced to NADPH through ICDH to be used in sulphate assimilation. Thus, in absence of NADH-dependent H_2 production, cells growing on sulphate needed to produce more lactate to produce higher amounts of NAD^+. This also agrees with a lower total NAD(H) pool observed in case of cells grown on sulphate. The hypothesis is further supported by a previous study, which demonstrated that under oxidative stress, *Pseudomonas fluorescens* upregulates *NAD^+ kinase* promoting synthesis of NADPH through synthesis of NADP (Singh et al., 2007). However, the gene coding for this enzyme has so far not been identified in *C. saccharolyticus*, but *NAD^+ kinase* is ubiquitous among bacterial genomes (Shi et al., 2009). The difference in *C. saccharolyticus*' behaviour under high P_{H_2} conditions with respect to the sulphur source is mediated via the differences in NAD(P)(H) pool. Thus, in addition to carbon source and growth rate (Willquist et al., 2011), the sulphur source also affects the metabolic flux distribution at the pyruvate node.

The overall hydrogen yield was reduced by about 40% when sulphate was used instead of cysteine (Fig. 3). Similarly, the overall acetate yield, the maximum specific H_2 productivity ($q_{H_2,Max}$) and overall accumulated H_2, were about 30–40% lower with sulphate (Fig. 3), but the overall lactate yield increased by about 5 times. No significant changes were observed in the overall biomass yield and the maximum specific glucose consumption rate ($q_{s,max}$, Fig. 3).

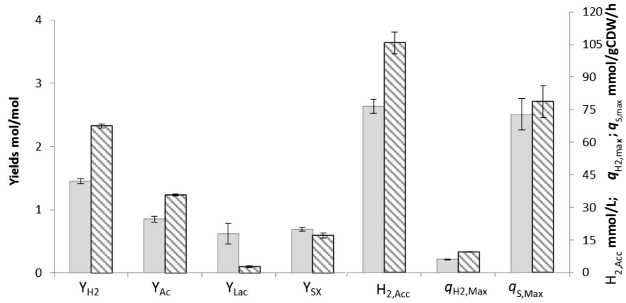

Fig. 3. Results of the batch cultures performed in absence of sparging in controlled bioreactors on media containing either $MgSO_4 \cdot 7H_2O$ (□) or cysteine.HCl (▨) as a source of sulfur. Y_{H_2}, Y_{Ac}, Y_{Lac}, and Y_{SX} represent molar yields of H_2, acetate, lactate and biomass per mol of hexose consumed, respectively. $H_{2,Acc}$, $q_{S,Max}$ and $q_{H_2,Max}$ represent total accumulated H_2 (mM), specific glucose consumption rate (mmol/gCDW/h) and maximum specific H_2 productivity (mmol/gCDW/h) respectively.

Table 4
Intracellular levels of ATP, PPi, NAD+ and NADH during batch cultures.

μmoles/gCDW	*Case* I ($MgSO_4 \cdot 6H_2O$)		*Case* II (Cysteine·HCl)	
	Exponential	Stationary	Exponential	Stationary
ATP	0.102 ± 0.025	0.036 ± 0.006	0.104 ± 0.02	0.055 ± 0.002
PPi	2.15 ± 0.04	1.44 ± 0.31	1.56 ± 0.55	0.92 ± 0
PPi/ATP*	25 ± 6	44 ± 11	17 ± 4	17 ± 1
NAD	0.85 ± 0.28	0.75 ± 0.31	1.57 ± 0.1	1.55 ± 0.09
NADH	0.063 ± 0.003	0.05 ± 0.01	0.16 ± 0.04	0.07 ± 0.01
NADH/NAD**	0.09 ± 0.03	0.07 ± 0.01	0.1 ± 0.03	0.04 ± 0.01

* Energy ratio.
** Redox ratio.

Table 5
Intracellular levels of NAD(P)(H) moiety (μM/gCDW), catabolic reduction charge (CRC) and anabolic reduction charge (ARC) during the continuous cultures.

Parameter	Feed C @ 0.2 *D*	Feed D @ 0.2 *D*	Feed E @ 0.2 *D*
NADH/NAD	0.39 ± 0.03	0.23 ± 0.1	0.31 ± 0.06
NAD(H)	0.8 ± 0.1	2.9 ± 0.6	2.0 ± 0.3
CRC	0.28 ± 0.03	0.18 ± 0.1	0.23 ± 0.06
NADPH/NADP	0.14 ± 0.05	0.25 ± 0.06	0.19 ± 0.03
NADP(H)	3.0 ± 0.1	4.6 ± 0.4	8.4 ± 1.9
ARC	0.12 ± 0.05	0.2 ± 0.06	0.16 ± 0.03

Intracellular levels of ATP and PPi were only determined for samples collected during batch cultures. Levels of both ATP and PPi were higher during the exponential phase than during the stationary phase of the corresponding cultures, independent of the sulphur source (Table 4). At exponential phase, no significant differences were observed in the levels of both ATP and PPi between both *Cases*. However, at stationary phase, the ATP concentration was significantly lower in cells using sulphate than for the culture using cysteine. In contrast, the PPi concentration was significantly higher in the culture using sulphate. The energy ratio (PPi/ATP) ratio were not significantly different between exponential and stationary phases for both the *Cases*. The energy ratio were at least an order of magnitude higher than the previously reported values (Bielen et al., 2010; Willquist et al., 2011), mainly due to the lower values of ATP observed during this study. Under saturated P_{H_2} conditions, the S-source does not have any significant effect on the intracellular levels of ATP and PPi (Table 4). PPi levels below 1 mM (intracellular concentration estimated from Table 4 using method described by Bielen et al., 2010), do not cause any significant inhibition of *lactate dehydrogenase* (LDH) activity (Willquist and van Niel, 2010). Moreover, at this non-inhibitory PPi concentration and a redox ratio lower than 0.2 (Table 4, Section 3.3), LDH is completely active (Willquist and van Niel, 2010). As both these conditions are satisfied during both exponential and stationary phases of batch cultures in the current study, an early onset of lactate production was observed in cultures using any of the sulphur source (Fig. 2B and C).

3.3. Levels of nicotinamide-containing coenzymes

During batch cultivations, no significant differences were observed in the intracellular NAD^+ concentrations between the exponential and stationary phase, regardless of the sulphur source used (Table 4). However, unlike with sulphate, with cysteine the NADH concentration was significantly higher in the exponential phase than the stationary phase. The NAD^+ concentration during both growth phases were twice as high in cultures with cysteine as in the corresponding growth phase of cultures using sulphate. A similar trend was observed for the NADH concentration in the exponential phase of the batch cultures. Although, the NADH concentrations in the stationary phase were indifferent regardless of the source of sulphur, the total NAD(H) pool was about twice higher in the cultures using cysteine than the cultures using sulphate. Only when using cysteine, a significant difference was observed in the NADH/NAD^+ (redox) ratio between the exponential and stationary phases. The redox ratio during stationary phase of the cultures using sulphate was significantly higher than that of culture using cysteine (Table 4).

In continuous cultures, the NAD(P)(H) levels were determined for the cultures operated at 0.2 h^{-1} and higher sulphur concentrations (Table 5). The total NADP(H) pool was significantly higher than the total NAD(H) level in the respective cultures. However, both the total NAD(H) and NADP(H) moieties were more than two times higher in the culture with cysteine (Feed E) than with sulphate (Feed C). The only trend observed between the different media from Feed C to Feed E was the increase of the NADP(H) pool (Table 5). However, no significant differences were observed in the redox ratios, the catabolite reduction charge (CRC) and the anabolic reduction charge (ARC) regardless of the source of sulphur used in the medium (Table 5).

Surprisingly, the redox ratio in S-limited culture was significantly higher than that of C-limited cultures reported previously (Willquist et al., 2011). The higher redox ratios (<0.3) in all chemostats with higher S-concentration would implicate stimulation of LDH (Willquist and van Niel, 2010), yet no significant lactate production was observed (Table 3). On the other hand, the decrease in NADP(H) moieties with increasing cysteine concentration indicate an increase in NADPH consumption required for sulphate assimilation (Table 5). Both the CRC and ARC were not affected by the S-source, of which the former was kept at an appropriate low value for effective catabolic fluxes (Table 5). Surprisingly, the ARC values were all well below 0.5, which might indicate starvation (Andersen and von Meyenburg, 1977). Since there was plenty of carbon substrate left during the steady states, one or other nutrient must be present at limited concentration. However, this merits further dedicated investigation.

3.4. Consequences for industrial applications

Lignocellulosic feedstock are increasingly being considered as raw materials for bio-hydrogen production at an industrial scale (Pawar and van Niel, 2013). Mild acid hydrolysis using sulphuric acid is one of the successful methods applied for the pre-treatment of these feedstock (Galbe and Zacchi, 2007). After the pre-treatment, neutralization of sulphuric acid with mild bases such as ammonium hydroxide can provide an inexpensive source of sulphur and nitrogen which can be assimilated by *C. saccharolyticus*. In addition, the finding that *C. saccharolyticus* does not release hazardous H_2S upon assimilation of sulphate adds to its industrial relevance. Furthermore, *C. saccharolyticus* displayed tolerance to high P_{H_2} when grown with sulphate. However, further research is needed to minimize the metabolic shift towards lactate. Nevertheless, use of sulphate may have significant techno-economic implications.

The differences in the assimilatory sulphur metabolism amongst *Caldicellulosiruptor* species can be exploited for creating inter-species dependence in defined co-cultures, e.g. in co-culture of *C. saccharolyticus* and *C. owensensis* using the medium with sulphate, the latter will depend on the former to provide S-containing amino acids through e.g. cell lysis. Also, addition of a chelating agent in the medium has allowed increasing the salt concentration in the cultivation medium without causing precipitation, which may enable *C. saccharolyticus* to convert higher amounts of substrate as a means to drive up H_2 productivities. However, since *C. saccharolyticus* is osmo-sensitive (Willquist et al., 2009), further studies are needed to evaluate the effect of the optimised medium on the H_2 productivity. This study also demonstrates, how the concentration of a key macro-nutrient, such as sulphur, can influence the critical D in a continuous culture. This can be significant in a process where reactors need to be operated at high D to obtain higher productivities. Hence, in a reactor system operating at high P_{H_2}, cysteine can still be added at a lower concentration to the medium as a reducing agent if required. However, *C. saccharolyticus* should then be genetically modified to prevent lactate production. Yet, a functioning protocol for genetic manipulations in *C. saccharolyticus* remains elusive.

4. Conclusions

C. saccharolyticus can assimilate sulphate efficiently in the absence of a reducing agent without any H_2S production. Although, *C. saccharolyticus* displayed tolerance to high P_{H_2} when grown with sulphate, it clearly performs better when cysteine is used instead. On the whole, sulphate can be a principle sulphur source for an economically viable and more sustainable bio-hydrogen process using majority of *Caldicellulosiruptor* species including *C. saccharolyticus*. The research and development of a thermophilic biohydrogen production process is still in its infancy, not the least, formulating a cheaper growth medium for *C. saccharolyticus* is of the essence for a cost-effective process.

Author's contribution

SSP planned the content of the article. SSP also planned and performed the experiments. EvN was involved in the planning of the experiments and supervised the process. EvN also critically reviewed the text. Both the authors have read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.07.059.

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Paper IV

Biofilm formation by designed co-cultures of *Caldicellulosiruptor* species as a means to improve hydrogen productivity

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Abstract

Background:
Caldicellulosiruptor species have gained reputation as being among the best microorganisms to produce hydrogen (H_2) due to possession of a combination of appropriate features. However, due to their low volumetric H_2 productivities (Q_{H2}), *Caldicellulosiruptor* species cannot be considered for any viable biohydrogen process yet. In this study, we evaluate biofilm forming potential of pure and co-cultures of *C. saccharolyticus* and *C. owensensis* in continuously stirred tank reactor (CSTR) and Up-flow anaerobic (UA) reactor. We also evaluate biofilms as a means to retain biomass in the reactor and its influence on Q_{H2}. Moreover, we explore the factors influencing the formation of biofilm.

Results:
Co-cultures of *C. saccharolyticus* and *C. owensensis* form substantially more biofilm than formed by *C. owensensis* alone. Biofilms improved substrate conversion in either of the reactor systems, but improved the Q_{H2} only in the UA reactor. When grown in the presence of each other's culture supernatant, both *C. saccharolyticus* and *C. owensensis* were positively influenced on their individual growth and H_2 production. Unlike the CSTR, UA reactors allowed retention of *C. saccharolyticus* and *C. owensensis* when subjected to very high substrate loading rates. In the UA reactor, maximum Q_{H2} (~20 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$) was obtained with granular sludge as carrier material. In the CSTR, stirring negatively affected biofilm formation. A clear correlation was observed between elevated (> 40 $\mu mol{\cdot}L^{-1}$) intracellular levels of the secondary messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) and biofilm formation.

Conclusions:
In co-cultures *C. saccharolyticus* fortified the trade of biofilm formation in *C. owensensis* mediated by elevated intracellular levels of c-di-GMP. These biofilms were effective in retaining biomass of both species in the reactor and improving Q_{H2} in an UA reactor using granular sludge as carrier material. This concept forms a basis for further optimizing the Q_{H2} at lab scale and beyond.

Keywords: *Caldicellulosiruptor saccharolyticus*, *Caldicellulosiruptor owensensis*, biohydrogen, co-culture, c-di-GMP, UA reactor, CSTR and volumetric H_2 productivity

1. Introduction

Amid the findings of vast reserves of shale oil and convenient negligence towards its (alleged) side-effects on the environment, the utopian world of 'hydrogen economy' still looks distant. One of the key bottlenecks is the unavailability of economical and eco-friendly ways of hydrogen production. Credible research is undergoing in developing sustainable processes producing hydrogen through electrolysis of water using wind and solar power (Delucchi & Jacobson, 2011). However, more alternatives are needed to complement these technologies. In this respect, fermentative hydrogen (biohydrogen) production at higher temperature, i.e. thermophilic biohydrogen production, using renewable biomass can be a viable option.

Caldicellulosiruptor species belong to a group of extremely thermophilic obligate anaerobes, possesses a natural ability of producing hydrogen from a wide range of mono-, di- and, oligo-saccharides and raw materials (de Vrije et al., 2009; de Vrije et al., 2010; Ivanova et al., 2009; Pawar et al., 2013; van de Werken et al., 2008). In addition to this, various other beneficial metabolic features enable the genus *Caldicellulosiruptor* as one of the best yet not ideal, group of bacteria with the natural ability to produce H_2 (Pawar & van Niel, 2013). Within this genus, *C. saccharolyticus* and *C. owensensis* are two of the best studied species; both known to produce H2 near the theoretical maximum of 4 $mol{\cdot}C6mol^{-1}$ (de Vrije et al., 2007; Zeidan & van Niel, 2010).

However, increasing Q_{H2} is one of the major challenges in developing a cost-effective biohydrogen process with *Caldicellulosiruptor* species. The Q_{H2} depends on various factors such as cell density, extent of substrate conversion and reactor configuration. The cell density can be increased by retaining more cells through different approaches, such as, immobilization, cell entrapment or cell retention. However immobilized or trapped cells can face mass transfer issues (Kumar & Das, 2001). In contrast, biofilms, are well-organized structures, and are inherent to cell retention (Dufour et al., 2010; Karatan & Watnick, 2009). Moreover, biofilms generally follow 'feed-and-bleed' cycles allowing cell growth, which can be significant for growth-dependent product formation (Karatan & Watnick, 2009). Among *Caldicellulosiruptor* species, *C. owensensis* has been previously reported to form biofilms (Peintner et al., 2010) mainly by flocculating at the bottom of the reactor. However, no further information could be found regarding the factor(s) leading to biofilm formation by *C. owensensis* (Peintner et al., 2010). Bis-(3′-5′)-cyclic dimeric

guanosine monophosphate (c-di-GMP) has been recognized as a ubiquitous secondary messenger in bacteria with multilayer control, i.e. at transcriptional, translational, and posttranslational level (Hengge, 2009; Jenal & Malone, 2006). The c-di-GMP is synthesized using 2 molecules of guanosine-5'-triphosphate (GTP) by the enzyme diguanylate cyclase (DGC) and is hydrolysed by the enzyme phosphodiesterase (PDE) (Jenal & Malone, 2006). Numerous studies have proven that high intracellular levels of c-di-GMP promote expression of extracellular-matrix related components needed for biofilm formation (Hengge, 2009; Jenal & Malone, 2006; Pérez-Mendoza et al., 2011).

So far, most of the research pertaining to biohydrogen, has been performed to investigate the physiological properties of H_2 producing microbes. These studies have mainly been performed in continuously stirred tank reactor (CSTR). However, CSTR systems do not allow cell retention. Hence, it is of paramount importance to evaluate alternative reactor types that can help retain the biomass. Several different reactor types, such as, packed bed reactor (Peintner et al., 2010), membrane bioreactor (Kim et al., 2011) , anaerobic sequencing blanket reactor (Prasertsan et al., 2009), trickle bed reactor (van Groenestijn et al., 2009) and up-flow anaerobic (UA) reactor (O-Thong et al., 2008) aiding cell retention have been reported to produce H_2 at higher rates. In fact, UA reactors are widely exploited for studies pertaining to biogas production. Their medium recirculation loop aids in achieving higher substrate conversion and also allows cells to adhere to the biofilms flocculated at the bottom of reactor. On the other hand, in case of the CSTR, using carriers has been reported to increase Q_{H2} by several folds (Koskinen et al., 2008).

In this study we aimed to evaluate the biofilm forming potential of *C. saccharolyticus* and *C. owensensis* in pure culture and also evaluate whether *C. owensensis* through biofilm formation aids *C. saccharolyticus* when cultivated in co-cultures. Furthermore, we report the intracellular levels of c-di-GMP in both the organisms and its relationship with biofilm formation. We also evaluate the potential of UA reactors in improving Q_{H2} compared to CSTR and whether carrier materials affect retaining the biomass and improving Q_{H2}.

2. Material and methods

2.1 Microorganism and its maintenance

C. saccharolyticus DSM 8903 and *C. owensensis* DSM 13100 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Routine subcultures and maintenance were conducted in 250-mL serum bottles containing 50-mL of a modified DSM 640 medium (Willquist et al., 2009) unless stated otherwise. Anoxic solutions of glucose, cysteine·HCl, and magnesium sulphate were autoclaved separately and added to the sterile medium at the required concentration. A 1000X concentrated vitamins solution was prepared as described previously (Zeidan & van Niel, 2010) and used in the growth medium at 1X concentration as a replacement to yeast extract. A 1000X concentrated trace element solution was prepared as described previously (Pawar & Van Niel, 2014).

2.2 Fermentation setup and culture medium

To study the effect of any excretion of *C. saccharolyticus* on the growth of *C. owensensis* and vice versa, batch cultures of each were performed in biological duplicates and previously collected cell-free culture supernatant of one organism was added into the batch medium of another prior to inoculation. The volume of supernatant added in each respective case was equivalent to that of occupying 1gCDW of the respective organism.

To study the effect of different reactor systems on biofilm formation and cell retention, *C. saccharolyticus* and *C. owensensis* were cultivated independently (pure culture) or together (co-culture) in two different reactor systems –i) continuously stirred tank reactor (CSTR) and ii) Up-flow anaerobic (UA) reactor (Table 1). To allow for biofilm formation and/or cell retention, co-cultures of *C. saccharolyticus* and *C. owensensis* were performed in both the reactor systems with K1-carriers (Catalogue # K1, AnoxKaldnes AB, Lund, Sweden). In case of the CSTR, co-cultures were performed with or without stirring, however, the pure cultures were performed only without stirring but with the K1-carriers (Table 1). In case of the UA reactor, the co-cultures were performed with and without using the granular sludge as packed bed, however, the pure cultures were performed only with granular sludge (Table 1).

All experiments were conducted in a jacketed, 3L (CSTR) or 1L (UA), equipped with an ADI 1025 Bio-Console and an ADI 1010 Bio-Controller (Applikon, Schiedam, The Netherlands) at a working volume of 1L (CSTR) or 0.85 L (UA), either in batch or continuous mode. The pH was maintained at 6.5 ± 0.1 at 70°C by automatic titration with 4M NaOH. The temperature was thermostatically kept at 70 ± 1°C. In case of the CSTR, a condenser with 5°C cooling water was fitted to the bioreactor's headplate and the stirring was kept at 250 rpm unless specified otherwise. UA reactor's top was fitted with a rubber cork inserted with a collection tube releasing the flue gas out of the reactor. During batch cultivations, culture samples were collected at different time intervals for monitoring growth and the culture supernatant was collected for analysis of glucose, acetic acid, lactic acid, propionic acid and ethanol. Gas samples were collected from the headspace to analyse levels of H_2 and CO_2. During continuous cultures, samples for c-di-GMP were collected at steady state. Batch cultures were performed in two independent biological replicates, whereas, for continuous cultures steady states were obtained in technical duplicates.

All the reactors were autoclaved with a base medium (BM) containing - KH_2PO_4 0.75 g; $K_2HPO_4{\cdot}2H_2O$ 1.5 g; NH_4Cl 0.9; yeast extract 1.0; resazurine 1 mg; 1000 X modified SL-10 1 mL. Solutions of glucose, 10 g/L for CSTR (Case A, B, C and D) and 20 g/L for UA reactors (Case E. F, G , H and I), cysteine·HCl, 0.25 g/L and $MgSO_4{\cdot}6H_2O$, 0.5 g/L were autoclaved and added separately prior to inoculation. The UA reactors with granular sludge (Case E, F and G) were autoclaved twice to eliminate the risk of methanogenic contaminants. Moreover, gas samples were regularly taken from the headspace of UA reactors to detect any traces of methane. Carriers were autoclaved separately and were added prior to inoculation. The granular sludge was obtained from methanogenic reactors treating municipal waste water. Inocula for each organism were prepared through a succession of at least 3 sub-cultivations prior to inoculation. In the case of co-cultures, inocula of each organism were grown separately.

For continuous cultivations, the bioreactor was started to be fed with fresh medium at the end of the logarithmic growth phase of the batch culture. Glucose was used as a primary substrate in all continuous experiments at an initial concentration of 10 g/L. Steady states were assessed after at least 5 volume changes based on the criteria of constant H_2 and CO_2 production rates and constant biomass concentration.

2.3 Analytical methods

Headspace samples were analysed for CO_2, H_2 and CH_4 by gas chromatography, using a dual channel Micro-GC (CP-4900; Varian, Micro gas chromatography, Middelburg, The Netherlands), as previously described (Zeidan & van Niel, 2010). The results were analysed with a Galaxie Chromatography Workstation (v.1.9.3.2). The optical density of the culture was measured at 620 nm (OD620) using a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). CDW was determined by filtration as previously described (Willquist & van Niel, 2010). Glucose, acetate, lactate, propionate and ethanol were analyzed by HPLC (Waters, Milford, MA, USA) on an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA) at 45°C, with 5 mM H_2SO_4 (0.6 ml·min^{-1}) as mobile phase. The column was equipped with a refractive index detector (RID-6A; Shimadzu, Kyoto, Japan).

2.4 Scanning electron microscopy of biofilm samples

Biofilm samples were scraped from the pH probe and/or carrier at the end of the cultivation (Case A) and were immediately stored overnight in glutaraldehyde solution (2-3%) to allow fixation. The samples were then stored with sodium cacodylate buffer (pH ~7) until further use. Few hours prior to scanning electron microscopy (SEM) imaging, samples were dried by first washing with ethanol solutions from 50 to 100% in series and then were subjected to 'critical point drying' using liquid CO_2. Subsequently, the dry biofilm samples were then glued on a stub and were sputter coated with gold/palladium and finally viewed under the SEM (Hitachi SU3500, Hitachi, Japan).

2.5 Determination of intracellular levels of c-di-GMP

During batch cultivations, 5 mL of culture samples were collected in quadruplets when cultures reached stationary phase. Similarly, in continuous cultures, 5 mL of culture samples were collected in quadruplets at steady state obtained under various conditions. All samples were collected on ice and were centrifuged immediately at 4000 rpm in a swinging bucket rotor at 4° C and were subsequently processed for the extraction of c-di- GMP. The extraction was performed as described by (Spangler et al., 2010) with the exception that in the final step, samples were dried by incubating overnight at approx. 50°C.

The quantification of c-di-GMP was performed as previously described (Spangler et al., 2010) but with the following modifications. The LC-separation were performed using isocratic conditions, 3.5% MeOH (A) and 96.5% 10 mM ammonium acetate in 0.1 % acetic acid (B) at 400 µl/min for 6.5 min. The internal standard XMP eluted after 3.1 min and c-di-GMP at 4.7 min. A wash program was run every 16 samples to ensure a robust analysis, in which 90% A was applied for 15 min before equilibrating the column for 20 min using the isocratic conditions. Standards, seven levels, ranging from 10 nM to 10 µM were included in the beginning and end of the sequence. The detection was performed using an Orbitrap-Velos Pro mass spectrometer (Thermo Scientific, Waltham, MA, USA) using the ESI in positive mode. Two scan events were applied: ion trap (ITMS) for quantification, including SRM on XMP (m/z 347/153 between 0 and 4 min) and c-di-GMP (m/z 691/540 between 4 and 6.5 min) and orbitrap fullscan (FTMS) for accurate mass identification, using a resolution of 30000.

2.6 Bioinformatics analysis for genes related to c-di-GMP

Genomes of *C. saccharolyticus* and *C. owensensis* were analysed to locate genes coding for DGC and PDE. All the information regarding genome sequences and corresponding annotations were retrieved from the Integrated Microbial Genomes (IMG, https://img.jgi.doe.gov/cgi-bin/w/main.cgi).

2.7 Population dynamics in the biofilm samples of co-cultures using qPCR

During all the co-culture experiments, 2 mL of culture samples were collected and immediately centrifuged and the cell pellets were stored at -20°C until further use. Similarly, sufficient amounts of biofilm samples were collected from the pH probe and from the reactor wall after the cultivations were ceased. The genomic DNA from the samples were extracted using Invitrogen's EasyDNA genomic DNA extraction kit (Catalogue # K1800-01) as per manufacturer's protocol and stored at -20°C until further use.

To determine the relative presence of *C. saccharolyticus* and *C. owensensis* in the co-cultures quantitative PCR (qPCR) assays were performed as described below. The 16S rDNA sequence was used as target for identification and quantification of each species. To design specific primers (Table 2), dissimilar regions were identified between target sequences using various sequence alignment tools available in the computer software - 'BioEdit' (Ibis Biosciences, Carlsbad, CA 92008). PCR

amplification and detection were performed in 'LightCycler® Nano' instrument (Roche Diagnostics, Mannheim, Germany). The PCR assay mixture (20 µL) contained – 1X ExTaq buffer, 1U TaKaRa ExTaq HS DNA polymerase, 4.5 mM $MgCl_2$, 0.2 mM dNTP (all from Th. Geyer GmbH, Germany), 2 µg BSA, 1X Eva green solution (Bioline GmbH, Germany), forward and reverse primers (each 0.5 µM, Table 2) and 4 µL of DNA template. For *C. saccharolyticus* the qPCR amplification protocol started with an initial denaturation at 95°C for 180 s, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 67°C for 10 s, and elongation and fluorescence acquisition at 72°C for 25 s. To confirm the absence of unspecific products melting-curve analysis was performed as follows - heating at 60°C for 60 s followed by an increase in temperature by 0.1 °C/s up to 97°C. Similar assays were performed for *C. owensensis*; albeit by changing the annealing temperature to 60°C. Quantification was performed using the method of 'absolute quantification' with the help of 'LightCycler® Nano' software *v*1.1. Pure genomic DNA samples (2.4 to 48 ng/µL) of each species were used in each run of the 'LightCycler® Nano' to establish a standard curve. Each run consisted of a blank assay with a PCR mixture containing dH_2O instead of DNA template. It also consisted a negative control assay with a PCR mixture containing the genomic DNA of other organism as a template. For a particular sample, the DNA concentration of each species was added together and then their relative fractions were determined.

2.8 Calculations

The Q_{H2} ($mmol{\cdot}L^{-1}{\cdot}h^{-1}$) and cumulative H_2 formation (CHF, $mmol{\cdot}L^{-1}$) were calculated in two different ways depending on the experimental design. All calculations were based on the ideal gas law and the H_2 and CO_2 concentrations in the headspace. For the cultures in CSTR, the calculations were based on the flow rate of the influent N_2 gas and the percentages of H_2 and CO_2 in the effluent gas, as no other gases were detected. Thus, Q_{H2} and CHF were calculated based on hydrogen concentration in the effluent gas and the flow rate of the effluent gas. For the experiments performed in the UA reactor the Q_{H2} was assumed to be twice the respective acetate productivities based on the stoichiometry (Shen et al., 2013). Product yields were calculated by determining moles of products formed per mole of glucose consumed. Biomass yield was calculated as moles of biomass formed per mole of glucose consumed. Carbon and redox balances were calculated as described previously (de Vrije et al., 2007).

3. Results

3.1 Results obtained from CSTR

Pure cultures in batch mode were tested for the influence of excretory metabolites from one species to another. For this reason, supernatant of one organism was added to the reactor of the other prior to inoculation. As control, both organisms were also grown in pure culture in absence of each other's supernatant. Batch cultures of both *C. saccharolyticus* and *C. owensensis* displayed significantly shorter lag phases when grown in the presence of each other's supernatant than in absence of it (Fig. 1A and B). Moreover, when exposed to each other's supernatant the cultures accumulated higher amounts of H_2 and biomass, and were less prone to cell lysis in the stationary phase (Fig. 1A and B). These are clear indications that both species might influence each other when in co-culture.

To evaluate the biofilm forming potential and its effect on biomass retention, Q_{H2}, substrate conversion rate and lactate formation by *C. saccharolyticus* and *C. owensensis*, experiments were performed in CSTR with or without K1-carriers (Cases A to D, Table 1). In continuous cultures performed in the CSTR, maximum Q_{H2} and maximum substrate conversion were obtained in Case A, whereas, maximum lactate productivity was observed in Case D (Fig. 2A, B and D). Cultures of Case A and D sustained growth at higher d (h^{-1}) than those of Case B and C (Fig. 2C). In case of Q_{H2}, no particular trend was observed for Case A with increasing d (h^{-1}), whereas, for Case B and C the Q_{H2} increased until d = 0.2 h^{-1} and then decreased. For Case D, Q_{H2} increased until d = 0.3 h^{-1} and then slightly decreased. The hydrogen yield was at its theoretical maximum only at low d (0.03-0.05 h^{-1}). Generally, for all the continuous cultures performed in the CSTR, the H_2 yield decreased with increasing d (h^{-1}), with the exception of Case A where it slightly increased at d > 0.3 h^{-1} (Fig. 2A). For Case A, the substrate conversion rate (SCR) increased with increasing substrate loading rate (SLR). For Cases B and C, the SCR increased with increasing SLR until d = 0.2 h^{-1} and then dropped. Similarly, for Case D, the SCR increased with increasing SLR until d = 0.3 h^{-1} and then decreased. For all the continuous cultures performed in the CSTR (Cases A to D) at 0.05 >d > 0.4 h^{-1}, in most cases the SLR was always higher than the SCR (Fig. 2B).

For all the continuous cultures performed in the CSTR, the planktonic biomass concentration generally decreased with increasing d (h^{-1}) (Fig. 2C). At any particular d (h^{-1}), Case A generally accumulated more planktonic biomass than Cases B, C or

D. Considering the pure cultures, *C. saccharolyticus* (Case C) showed higher biomass concentration compared to *C. owensensis* (Case D). Surprisingly, for Case B, the biomass yield suddenly increased at 0.3 h^{-1}, but was non-existent at higher d due to washout. No particular trend was observed in biomass yields with increasing d (h^{-1}) for Case A, C or D. The cultures of Cases A and D could not sustain growth at $d >$ 0.5 h^{-1}, whereas, cultures of Case B and C washed out at $d >$ 0.3 h^{-1} (Fig. 2C). Of the co-cultures, lactate production was only observed when the culture was not stirred (Case A), and increased with the d until 0.3 h^{-1} and decreased thereafter. Similarly, for the pure cultures, only *C. owensensis* (Case D) produced significant amounts of lactate which increased with the d until 0.3 h^{-1} and decreased thereafter. A similar trend was observed with the lactate yield for Cases A and D. Overall, the CSTR appeared to be an inappropriate system with respect to achieving higher SCR and Q_{H2}. Therefore, it was decided to evaluate another reactor type – the UA reactor.

3.2 Results obtained from continuous cultures in the UA reactor

Again, to evaluate the biofilm forming potential and its effect on biomass retention, Q_{H2}, substrate conversion rate and lactate formation by *C. saccharolyticus* and *C. owensensis*, experiments were performed in an UA reactor with either granular sludge or K1-carriers as carrier materials (Cases E to H, Table 1), or without any carrier (Case I, Table 1). The highest Q_{H2}, i.e. approximately 20 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$, was obtained in a co-culture with granular sludge at a d = 1.25 h^{-1} (Case E, Fig. 3A). The Q_{H2} of this culture increased steadily with increasing d (h^{-1}) and was higher than any other culture performed in the UA reactor at any particular d (h^{-1}). Other co-cultures, with and without K1-carriers, produced H_2 at significantly lower rates, but without any particular trend with increasing d (h^{-1}). On the other hand, the pure cultures of both organisms in the presence of granular sludge (Case F and G) produced H_2 at higher rates than the co-cultures without granular sludge (Case H and I, Fig. 3A). Among these pure cultures no significant differences were observed in Q_{H2} at any d (h^{-1}) except at 0.8 and 1.0 h^{-1}, where *C. owensensis* (Case G) displayed a slightly higher Q_{H2} (Fig. 3A). The H_2 yields were the highest for the co-culture with granular sludge compared to all other cultures at any particular d (h^{-1}) and generally varied between 2 to 3.3 mol of H_2/ mol of glucose consumed (Fig. 3A). The SCR in the UA reactor with granular sludge (Case E, F and G) generally increased with the SLR (at $d \leq 0.8$ h^{-1}) (Fig. 3B). Even though cultures with granular sludge (Case E, F and G) survived SLR values up to 140 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$, none of them displayed SCR more than 10 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$. At $d >$ 0.1 h^{-1}, cultures without granular sludge (Cases H and I) could not sustain growth at SLR values beyond approx. 90 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$and generally

displayed much lower SCR compared to cultures with granular sludge (Case E, F and G, Fig. 3B).

All liquid samples withdrawn from the granular sludge containing cultures (Case E, F and G) contained sludge granules, which made it difficult to determine the planktonic biomass concentration, thus no reliable data could be obtained. On the other hand, planktonic biomass concentration in cultures without granular sludge was very low (data not shown) as is evident from the low SCR values obtained in these cultures (Case H and I, Fig. 3B).

The highest lactate productivity was observed in *C. owensensis'* culture with granular sludge (Case G, Fig. 3C). At $d > 0.2\ h^{-1}$ both the pure cultures with granular sludge (Case F and G) displayed higher lactate productivity than the co-culture with (Case E) or without sludge (Case H and I). Of these co-cultures, the one without granular sludge (Case H and I) produced lactate at higher rates than the one with granular sludge (Case E). The lactate yields were lowest for the co-culture with granular sludge (Case E) at any particular d (h^{-1}). No significant differences in lactate yield were observed among the other cultures (Case F, G, H and I).

3.3 Biofilm formation by *Caldicellulosiruptor* species

No biofilm was observed during any of the batch cultures performed. In the continuous cultures, at $d > 0.2\ h^{-1}$ a substantial amount of flocculation was observed at the bottom of the CSTR in the co-culture when stirring was not applied (Case A, Additional file 1 and 2). In addition, in this culture at $d > 0.2\ h^{-1}$, biofilm was also observed on the reactor walls, pH probe and K1-carriers. In contrast, when stirring was applied (Case B), no biofilm was observed. Among the pure cultures, no biofilm was observed on the reactor wall, pH probe or K1-carriers in either of the Cases C and D. However, a biofilm in the form of flocculation of cells was observed in *C. owensensis*' culture for the entire duration (Case D). When viewed under SEM, the biofilm growing on the pH probe of the CSTR with co-culture (Case A) revealed distinct cells attached to each other with visible fibre-like structures (Fig. 4). Two different kinds of cell structures were observed, one as rod-shaped and unicellular form with dimensions 0.2-0.4 µm by 3-4 µm, whereas the other in a chain-like, multi-cellular structure with similar width, i.e. 0.2-0.4 µm, but variable length depending on the number of cells in a chain (Fig. 4).

The co-culture with sludge (Case E) displayed significant flocculation and biofilm

on the reactor wall which was especially pronounced at $d > 0.2\ h^{-1}$. Among the pure cultures, *C. owensensis'* culture with sludge (Case G) also displayed significant flocculation atop the sludge bed but hardly any biofilm was observed on the reactor walls. The co-cultures without sludge also displayed traces of biofilm on the reactor wall (Case H and I), however, no significant biofilm was observed on the K1-carriers (Case H).

3.4 Intracellular levels of c-di-GMP

The genomes of *C. saccharolyticus* and *C. owensensis* contain multiple genes coding for *diguanylate cyclase* (DGC), and *phosphodiesterase* (PDE) (Additional file 3). In batch cultures of *C. saccharolyticus* cells contained very low c-di-GMP levels compared to those observed in cells of *C. owensensis* (Fig. 5). Interestingly, when grown in the presence of each other's supernatant cells of *C. saccharolyticus* accumulated higher levels of c-di-GMP compared to those cells grown without the supernatant of a *C. owensensis* culture (Fig. 5). In contrast, the opposite trend was observed for *C. owensensis*. In continuous cultures, the co-culture without stirring (Case A) accumulated very low (<20 µM) levels of c-di-GMP at $d \leq 0.2\ h^{-1}$. However, at $d \geq 0.2\ h^{-1}$ the same culture accumulated at least 5-10 folds higher levels of c-di-GMP, albeit with no particular trend. Interestingly, in the co-culture without stirring (Case A) the levels of c-di-GMP appear to have increased when levels of residual sugar increased beyond 2 g/L (Fig. 6), without any particular pattern. In contrast, the co-culture with stirring accumulated very low (<30 µM) levels of c-di-GMP regardless of the d (h^{-1}). Among the pure cultures, cells of *C. owensensis* (Case D) accumulated similar levels to those observed in the co-culture without stirring (Case A) at $d \geq 0\ 2\ h^{-1}$, but approximately 10-fold higher levels than those observed in cells of *C. saccharolyticus* (Case C, Fig. 5).

Among the UA cultures, the co-culture without K1-carriers (Case I), except for d 0.2 and 0.4 h^{-1}, cells accumulated very low (<30 µM) c-di-GMP levels. The co-culture with K1-carriers (Case H) contained very low (<30 µM) c-di-GMP levels regardless of the d (h^{-1}) (Fig. 5). No samples were collected from cultures performed with sludge (Case E, F and G) due to contaminations from granular sludge.

3.5 Population dynamics in co-cultures of *C. saccharolyticus* and *C. owensensis*

In the co-culture without stirring performed in the CSTR (Case A), the biofilm on the pH probe consisted of *C. saccharolyticus* and *C. owensensis* in about 1:1 ratio (Fig. 7). However, in the same culture, the biofilm on the K1-carriers contained about 10-12 times more cells of *C. owensensis* than cells of *C. saccharolyticus*. Similarly, in the co-culture performed in the UA reactor (Case H), the biofilm on the K1-carriers contained the cell ratio of about 10:1 for *C. owensensis* compared to *C. saccharolyticus* (Fig. 7). No results could be obtained with samples collected from planktonic cells in any of the cultures possibly due to the low target DNA concentration.

4. Discussion

4.1 Effect of biofilm formation on Q_{H2}, substrate conversion and lactate formation

In a techno-economic analysis of a representative biohydrogen process, low Q_{H2} has been identified as a key bottleneck for making the process economically viable (Ljunggren et al., 2011). This study reports a higher Q_{H2} (approx. 20 $mmol \cdot L^{-1} \cdot h^{-1}$, Case E) than most of the previously obtained values in continuous cultures of *Caldicellulosiruptor* species (Bielen et al., 2013), but which is still about an order of magnitude lower than the maximum Q_{H2} ever reported for thermophilic hydrogen producers (O-Thong et al., 2008). Nevertheless, the highest maximum Q_{H2} in both these studies were obtained at very high d (> 1.0 h^{-1}), which may not be ideal for reasonable process economics (Ljunggren et al., 2011). Thus, further investigations are needed to determine the implications of high d (h^{-1}) on a biohydrogen process.

Numerous studies have asserted that biofilm formation improves substrate conversion leading to increased Q_{H2} (Kim et al., 2005; Koskinen et al., 2008; O-Thong et al., 2008). Similarly, in this study, formation of biofilm by co-cultures of *C. saccharolyticus* and *C. owensensis* improved the substrate conversion in CSTR as well as the UA reactor (Case A and E). However, it had a varied effect on Q_{H2}. In the UA reactor biofilm formation indeed improved Q_{H2}. In CSTR, however, improved substrate conversion was accompanied by increase in lactate production (Case A), which consequently subdued Q_{H2}. This abnormality of CSTR accumulating relatively higher amounts of reduced by-products, such as, lactate and

ethanol, than UA reactors (Case A and E) was also observed in a similar study comparing conversion of wheat straw hydrolysate using mixed culture in CSTR and UA reactor (Kongjan & Angelidaki, 2010). In the present study, the aforementioned abnormality may have occurred due to the presence of higher proportion of *C. owensensis* compared to *C. saccharolyticus* in planktonic phase at high d (> 0.2 h^{-1}) in CSTR. This hypothesis is supported by – i) regardless of the reactor system, *C. owensensis* produced higher amounts of lactate than *C. saccharolyticus* (Fig. 2D and 3C), ii) unlike the CSTR, the UA reactors inherently allow biomass retention thus perhaps higher fraction of cells of *C. saccharolyticus* were retained in the UA reactor compared to the CSTR when operated at higher d (h^{-1}).

4.2 Designed co-cultures vs. pure cultures

Regardless of the reactor system used, the co-cultures converted higher amounts of substrate and in the UA displayed higher Q_{H2} than the pure-culture of each species. This is in agreement with previous studies, where designed co-cultures of *C. saccharolyticus* and *C. kristjanssonii* showed higher H_2 yields than their pure-cultures (Zeidan et al., 2010). Similarly, a co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17 reported 2-fold higher Q_{H2} than either of their pure culture (Liu et al., 2008) even though they are of different genus.

Both *Caldicellulosiruptor* species performed better in batch growth in the presence of each other's supernatant, which clearly indicate that both species excrete compounds positively affecting the other one. A similar observation has been made for *C. saccharolyticus* excreting compound(s) that boosted the growth of *C. kristjansonii* (Zeidan et al., 2010). In fact, in co-culture *C. saccharolyticus* boosted the growth performance of *C. kristjansonii*, which can be interpreted as altruistic behaviour (Zeidan et al., 2010). In the current study, a similar behaviour was seen with *C. saccharolyticus* fortifying *C. owensensis'* ability to form biofilm. On its turn, *C. owensensis* showed altruistic behaviour by aiding *C. saccharolyticus* to take part in the biofilm formation (Fig. 6). This phenomenon is explained by 'kin selection theory' (Hamilton, 1964), according to which closely related species help each other to reproduce to pass its own genes to next generation even if indirectly. According to 'Hamilton's rule', higher relatedness (r) between the species and higher fitness benefit (b) to the beneficiary and lower fitness cost (c) to the 'altruist' will ensure better cooperation, i.e. $r*b - c > 0$ (Hamilton, 1964). This may explain why the co-culture of *C. saccharolyticus* and *C. kristjanssonii* reported higher H_2 yields (Zeidan et al., 2010) than any of the mixed cultures consisting microorganisms of various

genera ever reported. Indeed, another study argues simply that higher cooperation can be expected between highly related species (West et al., 2006).

Among the pure-cultures, both *C. saccharolyticus* and *C. owensensis* produced higher amounts of lactate than previously reported studies (de Vrije et al., 2007; Zeidan & van Niel, 2010) performed in similar conditions except that stirring was not applied for the cultures in this study. Obviously, the non-stirring condition led to oversaturation of H_2 and CO_2 in the culture leading to a shift in the metabolism (van Niel et al., 2003; Willquist et al., 2011). Finally, the observation of an unusual increase in biomass yield in the pure culture of *C. saccharolyticus* (Case C) near its critical d (0.3 h^{-1}) can be attributed to relatively higher energy spent by the culture on cell growth than product formation as a reaction to wash-out conditions at a high d (h^{-1}). A similar observation was reported in a previous study performed with *C. saccharolyticus* (Pawar & Van Niel, 2014). As far as we know this has not been described before in the literature and a clear rationale behind this phenomenon is lacking.

4.3 Effect of reactor system and culture conditions

In UA reactors, only granular sludge provided a supporting bed to the flocculating biofilms of *C. owensensis* and *C. saccharolyticus*. This explains the very low Q_{H2} observed in the UA reactor without granular sludge. Similar results were obtained in a previous study performed with *Thermoanaerobacterium thermosaccharolyticum* PSU-2 (O-Thong et al., 2008). However, despite its benefits, the risk of contamination from hydrogenotrophic methanogens threatens the stability of UA reactors when granular sludge is used. Perhaps, porous glass beads may be a viable alternative carrier. A recent study reported an increase in Q_{H2} and H_2 yield by 70% and 30%, respectively, when cells of *Thermotoga neapolitana* were immobilized on porous glass beads in CSTR (Ngo & Bui, 2013).

Although, higher Q_{H2} (>15 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$) is desirable for better process economics, a higher H_2 yield (>3 $mol{\cdot}mol^{-1}$) can certainly contribute to improving the process economics when relatively expensive raw materials are used. In that respect, when the results obtained in this study are compared, UA reactors appear to offer a process alternative to achieve high Q_{H2} and yield (Fig. 8). The CSTR, on the other hand, seems to have a boundary value around 10 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$for the Q_{H2} regardless of the H_2 yield (Fig. 8).

The UA reactor allowed d (h^{-1}) well beyond the maximum specific growth rates of *C. saccharolyticus* and *C. owensensis* in pure and co-cultures underlining the ability of UA reactors to retain the biomass of these species.

4.4 Biofilm and intracellular levels of c-di-GMP

A clear correlation was observed between the high intracellular c-di-GMP levels (>40 µM) and the stage of a particular culture initiating a biofilm. Although, the samples were collected from planktonic biomass and not the biofilm itself, since the biofilms go through feed-and-bleed cycles, the planktonic cells can be assumed to be representative of the cells in the biofilm. Conversely, in the absence of any biofilm, very low c-di-GMP levels were observed when stirring was applied in continuous cultures in the CSTR (Case B). However, batch cultures of *C. owensensis* accumulated high levels of c-di-GMP but no biofilm was observed, perhaps due to the stirring. Moreover, c-di-GMP levels in co-culture performed without stirring (Case A) increased as the concentration of residual sugar increased beyond 2 $g{\cdot}L^{-1}$ (Fig. 6). This may be because of a combination of – i) flocculating cells of *C. owensensis* at the bottom of the CSTR did not have access to the influent feed being dropped from the top of the CSTR, and ii) cells of *C. saccharolyticus* dominating the planktonic phase, consumed most of the substrate until a d of 0.1 h^{-1}, after which the residual concentration increased beyond 2 g/L (Fig. 6). Beyond that point the glucose gradient may have reached *C. owensensis* at the bottom allowing in the development of biofilms at $d \geq 0.2\ h^{-1}$. Thus, it can be argued, that if the co-cultures were performed at high substrate concentration, biofilm could have been obtained even at $d < 0.2\ h^{-1}$. This knowledge may help in achieving SLRs as well as biofilms at low d (h^{-1}) similar to those obtained at high d (h^{-1}) in this study. However, the vulnerability of *C. saccharolyticus* to high osmotic pressure limits the option of performing cultures using feed with high substrate concentration (Willquist et al., 2009). Alternatively, a reactor system such as an UA reactor which feeds the influent from bottom may also be more appropriate, as shown in the present study.

Although, *C. saccharolyticus* possesses genes required for synthesis of c-di-GMP, its intracellular levels are well below the critical level (40 µM). This perhaps explains the inability of *C. saccharolyticus* to form biofilm independent of *C. owensensis*. Arguably, over expression of DGC may elevate the levels of c-di-GMP in *C. saccharolyticus* allowing biofilm formation. Thus, encouraging *C. saccharolyticus* to

form biofilms on its own may provide a better alternative to its co-culture with *C. owensensis*, considering the propensity of the latter to produce lactate and ethanol.

5. Conclusions

Only when grown together in co-culture, *C. saccharolyticus* and *C. owensensis* form substantial amounts of biofilm improving substrate conversion and Q_{H2}. Thus, such a constructed co-culture is an effective means to be exploited in any bioreactor designed for biomass retention such as UA reactor. Indeed, UA reactor allows retention of *C. saccharolyticus* and *C. owensensis* when subjected to very high substrate loading rates, improving substrate conversion and Q_{H2}. Granular sludge showed superior support to biofilm formation in the UA reactor. However, as sludge can be a potential source of methanogenic contaminants, it either needs proper pre-treatment or more suitable alternatives should be found. Elevated intracellular levels of c-di-GMP are clearly linked to biofilm formation by *C. saccharolyticus* and *C. owensensis*. The maximum Q_{H2} obtained in this study was obtained at very high d (h^{-1}) which may not be ideal for a reasonable process economics. Alternatively, a biofilm forming pure or co-cultures of *Caldicellulosiruptor* species, which can withstand feed containing high substrate concentration can be operated at a reasonably low d (h^{-1}), which will allow similar substrate loading rates to that obtained in this study at high d (h^{-1}). The way forward to industrial application is to further exploit the concept of this designed co-culture in UA-type reactors using granular sludge-type of carriers for obtaining higher volumetric hydrogen productivities.

Fig. 1 Growth and H_2 accumulation by *C. saccharolyticus* and *C. owensensis* in pH-controlled batch fermentations: in the presence (solid green line) and absence (dotted blue line) of each other's supernatant, *C. saccharolyticus* (A), *C. owensensis* (B), OD measured at 620 nm when grown with supernatant (open circles) and without supernatant (filled circles); H_2 accumulation when grown with supernatant (open diamonds) and without supernatant (filled diamonds).

Fig. 2 Results of the continuous cultures of *C. saccharolyticus* and *C. owensensis* performed in CSTR. (A) Q_{H2}, line graph ($mmol \cdot L^{-1} \cdot h^{-1}$) and H_2 yield, bar graph ($mol \cdot mol^{-1}$), (B) substrate conversion rate ($mmol \cdot L^{-1} \cdot h^{-1}$); (C) OD measured at 620 nm, line graph and, biomass yield, bar graph ($mol \cdot mol^{-1}$) from planktonic phase; and (D) lactate productivity ($mmol \cdot L^{-1} \cdot h^{-1}$), line graph and, lactate yield ($mol \cdot mol^{-1}$), bar graph. Case A (open circles, filled bar); Case B (filled circles, dotted bar); Case C (open triangles, bar with vertical lines) and, Case D (filled triangles, bar with skewed lines). Substrate loading rate (solid black line with open squares)

Fig. 3 Results of the continuous cultures of *C. saccharolyticus* and *C. owensensis* performed in UA reactors. (A) Q_{H2}, line graph ($mmol \cdot L^{-1} \cdot h^{-1}$) and H_2 yield, bar graph ($mol \cdot mol^{-1}$), (B) substrate conversion rate and substrate loading rate ($mmol \cdot L^{-1} \cdot h^{-1}$); and (C) lactate productivity ($mmol \cdot L^{-1} \cdot h^{-1}$), line graph and, lactate yield ($mol \cdot mol^{-1}$), bar graph. Case E (open circles, filled bar); Case F (open squares, open bar); Case G (open triangles, bar with vertical lines), Case H (filled triangles, dotted bar) and, Case I (filled squares, bar with horizontal lines). Substrate loading rate (solid line with open squares).

Fig. 4 SEM image of a biofilm obtained from the pH probe from the co-culture (Case A).

Fig. 5. Intracellular levels of c-di-GMP in batch and continuous cultures performed in CSTR and UA reactors.

Batch cultures without supernatant: *C. saccharolyticus* (filled circle, green), *C. owensensis* (filled square, green); batch cultures with each other's supernatant: *C. saccharolyticus* (open circle, blue), *C. owensensis* (open square, green); Continuous cultures: Case A (filled triangle, red), Case B (open triangle, red), Case C (filled circle, yellow), Case D (open circle, yellow), Case I (filled diamond, black) and Case H (open diamond, black). For continuous cultures, the values on X-axis represent d (h^{-1}) at which the sample was collected.

Fig. 6 Correlation between intracellular c-di-GMP levels and residual sugar concentration in the co-culture (Case A).

Fig. 7 Fraction of *C. saccharolyticus* and *C. owensensis* in biofilm samples (Case A and H). *C. owensensis* (filled, blue) and *C. saccharolyticus* (horizontal lines, green), values on X-axis represent the source of the biofilm sample with respect to reactor system and the carrier.

Fig. 8 The correlation between Q_{H2} and H_2 yield in co-cultures (Case A and E). Q_{H2} ($mmol \cdot L^{-1} \cdot h^{-1}$), H_2 yield ($mol \cdot mol^{-1}$). Case A (filled circle, blue), Case E (open circle, red); the encircled data point represent the best case scenario where both Q_{H2} and H_2 yield are reasonably high.

Additional File 1 The planktonic biomass in the co-culture without stirring (Case A). The boxes filled with different colours represent a particular d (h^{-1}).

Additional File 2 A short-film showing the biofilm in action (Case A). https://www.youtube.com/watch?v=TVWWefm3fmU&feature=youtu.be

6. Abbreviations

d	dilution rate; h^{-1}
Q_{H2}	volumetric H_2 productivity; $mmol \cdot L^{-1} \cdot h^{-1}$
SCR	substrate conversion rate; $mmol \cdot L^{-1} \cdot h^{-1}$
SLR	substrate loading rate; $mmol \cdot L^{-1} \cdot h^{-1}$
CSTR	Continuously stirred tank reactor
UA	Up-flow anaerobic
c-di-GMP	bis-(3′-5′)-cyclic dimeric guanosine monophosphate
DGC	*diguanylate cyclase*
PDE	*phosphodiesterase*
CHF	cumulative H_2 formation, $mmol \cdot L^{-1}$

7. Author's contribution

SSP planned the content of the article and, planned and performed the experiments. TV assisted SSP in some of the batch and continuous cultures. CG optimized and performed the analysis of c-di-GMP and also contributed with writing related to c-di-GMP. EvN was involved in the planning of the experiments and supervised the process. EvN also critically reviewed the text. All the authors have read and approved the manuscript.

8. Competing interests

The authors declare that they have no competing interests.

9. Acknowledgement

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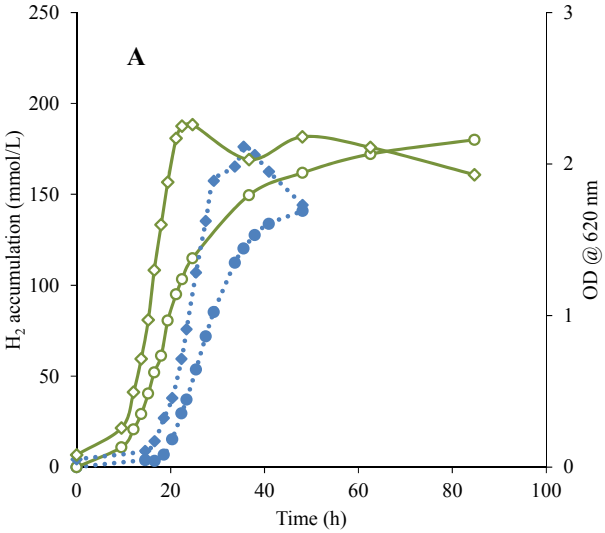
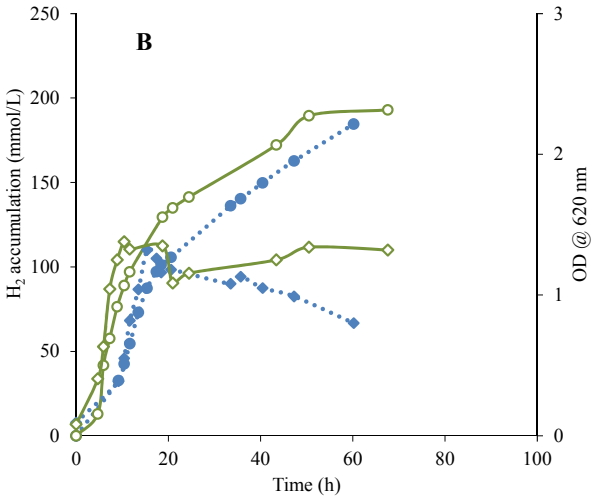
Table 1 Various cultivation conditions applied during this study

Name	Cultivation condition
Case A	Co-culture in CSTR w/o stirring w/ carriers
Case B	Co-culture in CSTR w/ stirring w/o carriers
Case C	*C. saccharolyticus* w/o stirring w/ carriers
Case D	*C. owensensis* w/o stirring w/ carriers
Case E	Co-culture in UA reactor w/ sludge
Case F	*C. saccharolyticus* in UA reactor w/ sludge
Case G	*C. owensensis* in UA reactor w/sludge
Case H	Co-culture in UA reactor w/o sludge w/ carriers
Case I	Co-culture in UA reactor w/o sludge w/o carriers

Table 2 Primers used in this study. Primers for *C. saccharolyticus* were obtained from a previous study (Zeidan et al., 2010).

Organism (Locus tag)	Primer	Sequence	Product (bp)
C. saccharolyticus (Csac_R0001)	F_R0001	GGTGCGTAGGCGGCTATGCG	448
	R_R0001	CCCACCCTTTCGGGCAGGTC	
C. owensensis (Calow_R0003)	F_R0003	GCTAAGCGGATGGGGGAAACT	582
	R_R0003	CTGGCAGTGTTGAACGC	


A
250
200
150
100
50
0
3
2
1
0
0
20
40
60
80
100
Time (h)
H2 accumulation (mmol/L)
OD @ 620 nm


B
250
200
150
100
50
0
3
2
1
0
0
20
40
60
80
100
Time (h)
H2 accumulation (mmol/L)
OD @ 620 nm

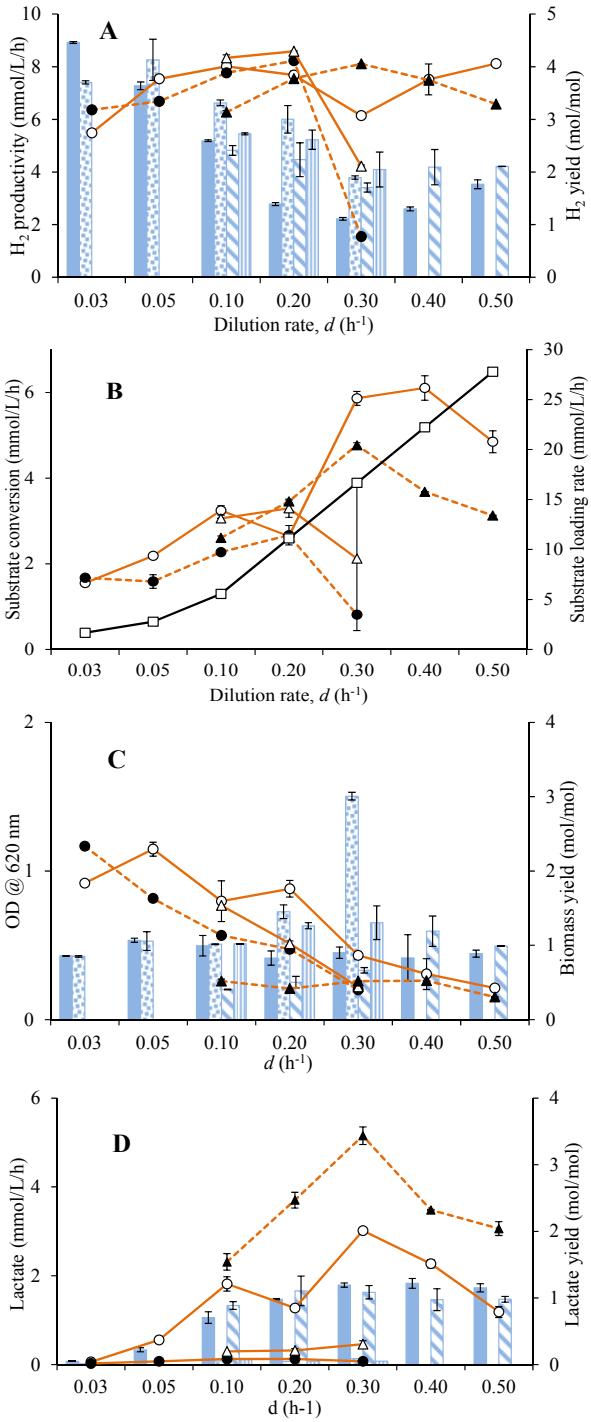


Fig. 2 A, B, C, and D

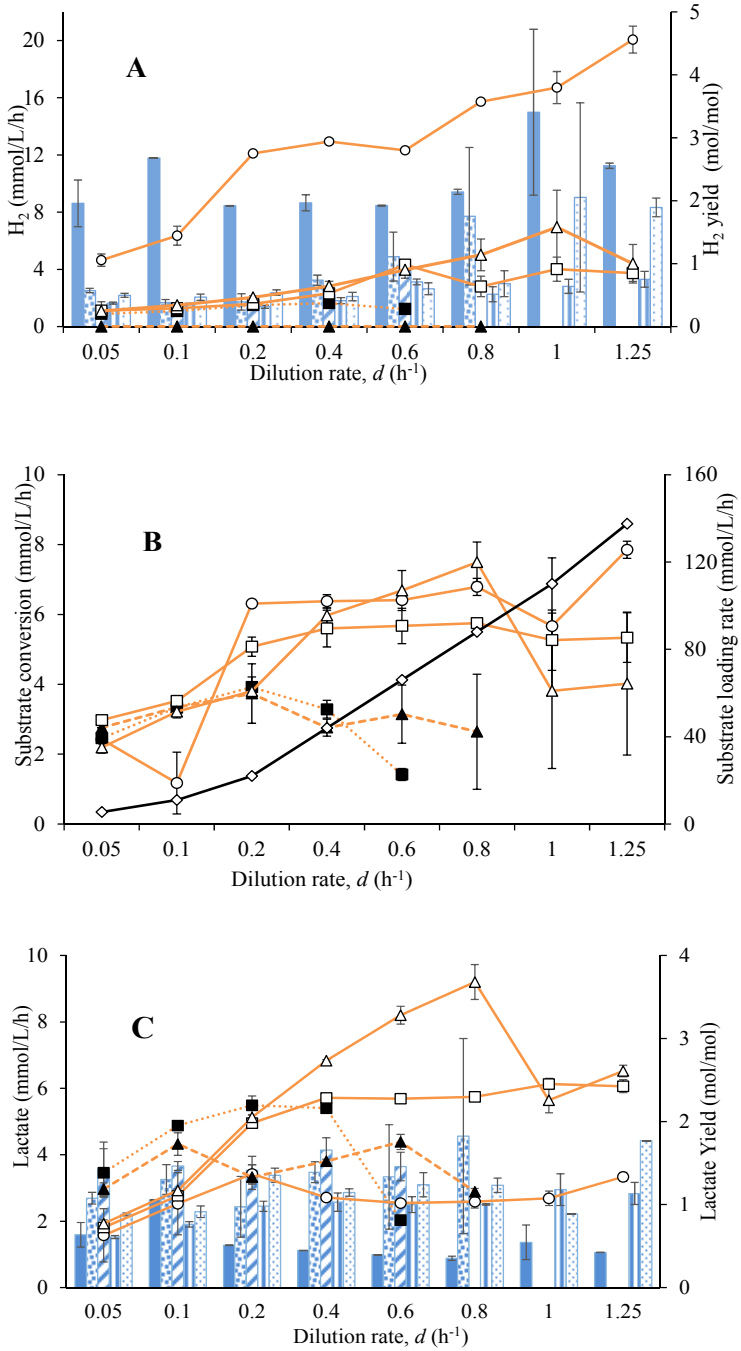

A
H_2 (mmol/L/h)
H_2 yield (mol/mol)
Dilution rate, d (h^{-1})
B
Substrate conversion (mmol/L/h)
Substrate loading rate (mmol/L/h)
Dilution rate, d (h^{-1})
C
Lactate (mmol/L/h)
Lactate Yield (mol/mol)
Dilution rate, d (h^{-1})

Fig. 3 A, B, and C

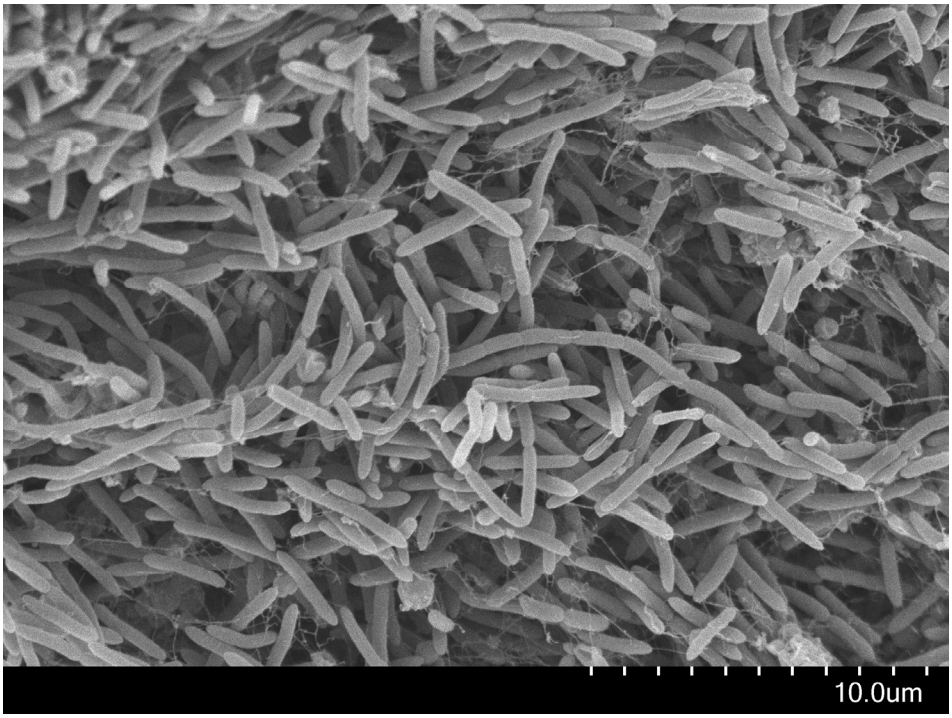


Fig. 4

Intracellular levels of c-di-GMP (μM)
0
50
100
150
200
250
300
350
0,03
0,05
0,1
0,2
0,3
0,4
0,5
0,03
0,05
0,1
0,2
0,3
0,1
0,2
0,3
0,1
0,2
0,3
0,4
0,5
0,1
0,2
0,4
0,6
0,03
0,05
0,1
0,2
0,4
0,8

Fig. 5

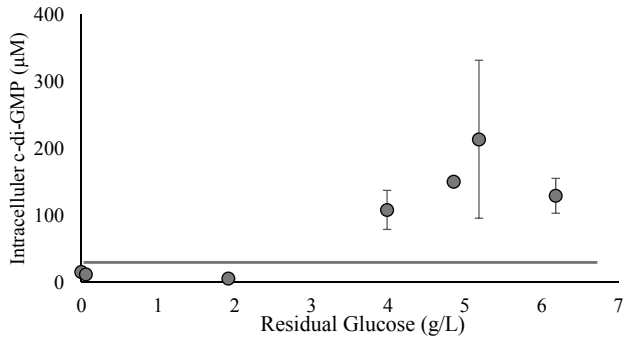


Fig. **6**

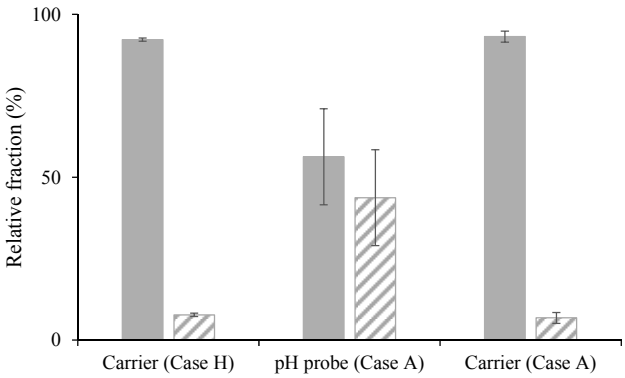


Fig. 7

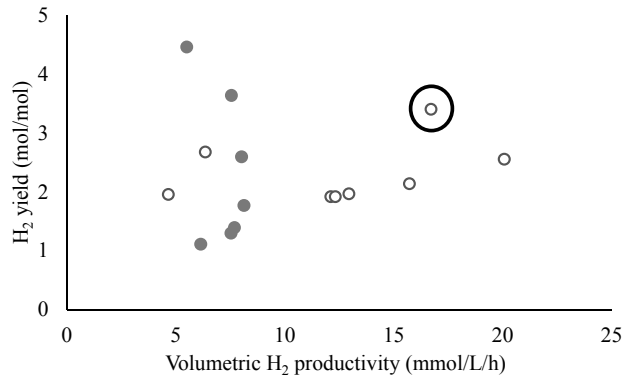
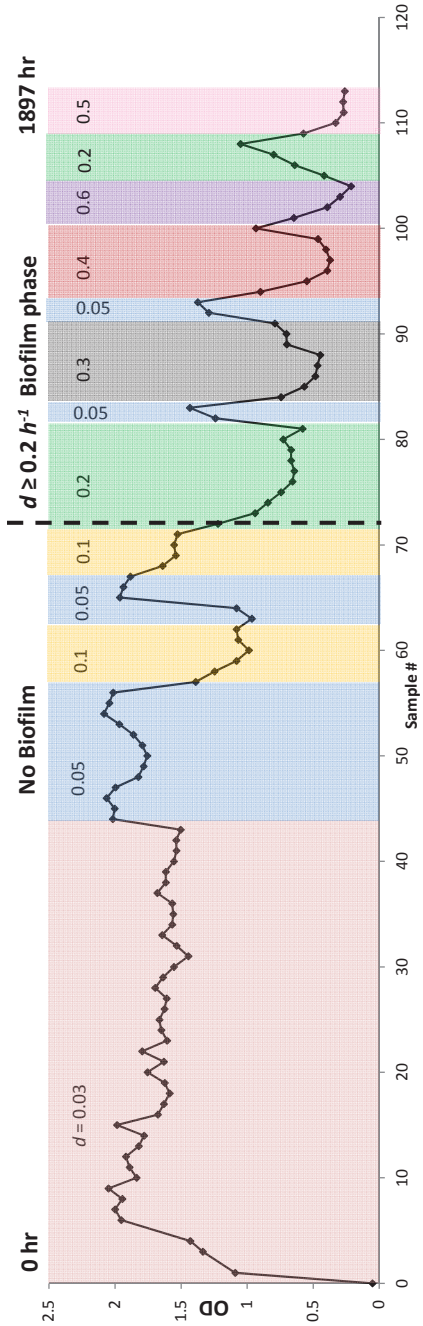


Fig. **8**



Additional file 1

Paper V

Development of osmotolerant strains of *Caldicellulosiruptor saccharolyticus* as a means to improve hydrogen productivity

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Abstract

Caldicellulosiruptor saccharolyticus is a well-known, Gram-positive, extremely thermophilic and cellulolytic microorganism with a natural ability to produce hydrogen (H_2). However, to become fit for commercial exploitation, it's volumetric H_2 productivity (Q_{H2}) needs to be improved further. Improvement in the substrate conversion rates (SCR) by *C. saccharolyticus* may enhance its Q_{H2}. Since it is sensitive to higher substrate and product concentrations, several osmotolerant strains of *C. saccharolyticus* viz., *C. saccharolyticus* G10 and *C. saccharolyticus* AG6 were developed through evolutionary engineering. The G10 strain displayed stable growth in a medium containing approx. 90 g/L of glucose, and the strain AG6 grew successfully in a medium containing approx. 13.2 g/L of sodium acetate and 30 g/L of glucose. An ancestor of the strain G10, *C. saccharolyticus* G5 achieved the highest Q_{H2}, i.e. 20.7 mmol/L/h, ever reported for continuous cultures performed in CSTR with *C. saccharolyticus*. The transcriptomic analysis of the evolved strains suggests that transposition of insertion sequence may have assisted in the development of beneficial mutations leading to adaptation. Furthermore, it suggests that the optimization of sulphate and ammonium concentrations in the growth medium of *C. saccharolyticus* may improve the Q_{H2} further. In conclusion, evolutionary engineering proves to be a reliable method for the development of osmotolerant strains of *C. saccharolyticus*. However, further characterization and development of these strains at genetic level may be necessary before considering any industrial application.

Keywords: *Caldicellulosiruptor saccharolyticus*, substrate conversion rate, volumetric hydrogen productivity, evolutionary engineering, CSTR, transposition and insertion sequence

1. Introduction

The latest report on climate change by the Intergovernmental Panel on Climate Change (IPCC) asserts the increase in the atmospheric levels of greenhouse gases is the main reason behind the global warming (Stocker, 2013). Although, other factors, such as volcanic eruptions, changes in solar luminosity and variations in Earth's orbit around the Sun, can also cause changes in Earth's climate (Solomon, 2007), the only controllable factor directly responsible for climate change is the use of fossil fuels in human activities. Thus, to minimize further CO_2 emissions, environmental friendly and renewable sources of energy need to be found. Next to other alternative sources, such as solar, wind and geo-thermal energy, waste biomass also possesses huge potential as an indigenous and renewable energy source (Klass, 1998).

Due to its CO_2-free combustion and high energy density among other desirable properties as a fuel, hydrogen (H_2) is touted as a fuel of the future (Balat, 2008; Ingersoll, 1996; Sequeira & Santos, 2010). Several thermophilic microorganisms are capable of producing H_2 using various kinds of renewable biomass (Kengen et al., 2009; Pawar & van Niel, 2013). However, the members of the genus *Caldicellulosiruptor*, in particular, are gaining recognition as being one of the most efficient thermophilic H_2 producers (Pawar & van Niel, 2013).

Caldicellulosiruptor saccharolyticus is an extremely thermophilic obligate anaerobe, capable of producing hydrogen from a variety of mono-, di- and, oligo-saccharides contained in different renewable biomass (de Vrije et al., 2009; de Vrije et al., 2010; Ivanova et al., 2009; Pawar et al., 2013; van de Werken et al., 2008). Moreover, *C. saccharolyticus* has the ability to sustain growth and H_2 production in the absence of any means of H_2 removal, such as sparging (Willquist et al., 2011) and/or stirring (Pawar et al., 2014). Furthermore, its ability to grow in a chemically defined medium (Willquist & van Niel, 2012) and to assimilate inorganic sulphate salt as a sole source of sulphur (Pawar & van Niel, 2014) enables a reduction in the cost of nutrient requirements. However, despite such desirable properties, increasing the volumetric H_2 productivity (Q_{H2}) is one of the major bottlenecks to developing a cost-effective biohydrogen process with *C. saccharolyticus* (Ljunggren et al., 2011; Ljunggren & Zacchi, 2010). Indeed, even after numerous attempts of improving the Q_{H2} of *C. saccharolyticus*, it still remains at least a quarter of magnitude lower than the desired value (Bielen et al., 2013).

The Q_{H2} can be improved by increasing the biomass density and/or extent of substrate conversion. The latter can be improved by increasing the substrate

concentration in the medium. However, *C. saccharolyticus* is unable to grow at solute concentration higher than 400 - 425 mM (van Niel et al., 2003) . Moreover, when the osmolality of the medium increases more than 220 mOsmol/Kg H_2O, its central carbon metabolism undergoes a metabolic shift to produce lactate and ethanol instead of H_2 (Willquist et al., 2009).

Therefore, in this study we aimed to develop osmotolerant strains of *C. saccharolyticus* capable of growing in high sugar and/or product concentrations. We also performed transcriptomics of these strains when cultured in a medium with high or low osmolality. Finally, we studied the extent of substrate conversion by one of the osmotolerant strains by growing it in a modified medium with high nutrient concentrations including the sugars.

2. Material and methods

2.1 Microorganism and its maintenance

C. saccharolyticus DSM 8903 and *C. owensensis* DSM 13100 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Routine subcultures and maintenance were conducted in 250-mL serum bottles containing 50-mL of a modified DSM 640 medium (Willquist et al., 2009) unless stated otherwise. Anoxic solutions of glucose, cysteine·HCl, and magnesium sulphate were autoclaved separately and added to the sterile medium at the required concentration. A 1000X concentrated vitamins solution was prepared as described previously (Zeidan & van Niel, 2010) and used in the growth medium at 1X concentration as a replacement to yeast extract. A 1000X concentrated trace element solution (modified SL-10) was prepared as described previously (Pawar & van Niel, 2014).

2.2 Evolutionary engineering of *C. saccharolyticus*

Evolutionary engineering of *C. saccharolyticus* was performed by adapting it to a medium with step-wise increase in its osmotic pressure using two different agents – i) glucose (Table 1) and ii) combination of sodium acetate (NaAc) + glucose (Table 2). The adaptation experiments were performed in serum flasks. Stability of the strains was established at each stage by confirming decrease in their generation time

to less than 4 hours (same as wild-type (WT) *C. saccharolyticus* in a low-osmotic medium) for at least three repeated batches and then were transferred to the subsequent stage to continue adaptation (Table 1 and 2). The strains obtained at each stage of the experiment were given a specific alphanumeric designation based on the type and concentration of the agent they were adapted to. The stability of the strains was evaluated by growing them for at least 5 successive batch cultivations (20-25 generations) in a low-osmotic culture medium and then transferring them to their respective high-osmotic medium (e.g. *C. saccharolyticus* G10 was transferred to a medium containing 100 g/L of glucose).

For all the evolutionary engineering experiments the preparation of anaerobic flasks was performed as follows: defined medium (DM) containing (per Liter): KH_2PO_4, 0.75 g; $K_2HPO_4{\cdot}2H_2O$, 1.5 g; NH_4Cl, 0.9; resazurine, 1 mg; 1000 X modified SL-10, 1 mL, without the carbon source, cysteine·HCl, and vitamins, was added to the flasks and thereafter, the flasks were sealed with butyl stoppers and aluminium crimps. Similarly, serum flasks containing concentrated solutions of each of the remaining medium components were prepared. Subsequently, the headspaces of the flasks were flushed with N_2 and were autoclaved. The vitamin solution was filter sterilized using 0.2 µm sterile AcroCap™ filters. The flasks containing a complete culture medium were then inoculated with an appropriate *C. saccharolyticus* strain.

Table 1 Development of *C. saccharolyticus* G10

Stage	Glucose (mM)	Final (mM)*	Generations	Strain
1	55	210	15	G1
2	111	265	40	G2
3	167	322	25	G3
4	222	377	25	G4
5	278	432	75	G5
6	334	488	>100	G6
7	389	543	>100	G7
8	555	709	>200	G10

*Final molarity of the medium

Table 2 Development of *C. saccharolyticus* AG6

Stage	NaAc (mM)	Glucose (mM)	Final (mM)*	Generations	Strain
1	55	22	231	40	AG1
2	110	22	286	>100	AG2
3	164	22	340	>100	AG3
5	164	55	373	>100	AG4
6	164	111	429	>100	AG5
7	164	167	485	>200	AG6

* Final molarity of the medium

2.2 Fermentation setup and culture medium

All experiments were conducted in a jacketed CSTR equipped with an ADI 1025 Bio-Console and an ADI 1010 Bio-Controller (Applikon, Schiedam, The Netherlands) at a working volume of 1L (CSTR) or 0.85 L (UA), in continuous mode. The pH was maintained at 6.5 ± 0.1 at 70°C by automatic titration with 4M NaOH. The temperature was thermostatically kept at 70 ± 1°C. A condenser with 5°C cooling water was fitted to the bioreactor's headplate. Gas samples were collected from the headspace to analyse levels of H_2 and CO_2 regularly and also at the steady state. Similarly, liquid samples were collected for determination of growth and quantification of soluble metabolites. Samples for cell dry weight and RNA extraction were collected only at steady state. Steady states were obtained in technical duplicates.

All the reactors were autoclaved with DM medium, solutions of glucose 10 g/L, cysteine·HCl, 0.25 g/L and $MgSO_4 \cdot 6H_2O$, 0.5 g/L were autoclaved and added separately prior to inoculation. The reactors were continuously sparged with N_2 at 9 L/h and stirred at 250 rpm. The reactors were inoculated with cells of relevant strains pre-grown on a DM medium containing 10 g/L of glucose for three successive batches in serum flasks. The cells from third sub-cultivations were then inoculated to the respective reactors. The reactors were started to be fed with fresh medium at the end of the logarithmic growth phase of their respective batch cultures. The composition of the feed used in all the chemostats was similar to the one used for the batch (unless specified otherwise) cultivation except for the carbon source, which varied with respect to the strain and cultivation condition (Table 3). In case of *C.*

saccharolyticus G5, the feed contained (per Litre) - $(NH_4)_2SO_4$, 0.5; NH_4Cl, 1; $MgCl_2{\cdot}7H_20$, 0.45; KH_2PO_4, 1 and an additional 1 mL each of modified SL-10 and vitamin solution was added per Litre of the feed. Steady states were assessed after at least 5 volume changes based on the criteria of constant H_2 and CO_2 production rates and constant biomass concentration.

Table 3 Composition of various feed at low and high osmotic condition (g/L).

Strain	Low	High
G10	G, 10	G, 90 (667±20)†
G3Ac3	G, 10	G, 30; Ac, 13.27 (547±20)†
G5	-	G, 30; X, 15; A, 4.5
WT	G, 10	-

* G, glucose, Ac, sodium acetate, X, xylose, A, arabinose; †, osmolality mOsmol/Kg·H_2O)

2.3 Analytical methods

Headspace samples were analysed for CO_2 and H_2 by gas chromatography, using a dual channel Micro-GC (CP-4900; Varian, Micro gas chromatography, Middelburg, The Netherlands), as previously described (Zeidan & van Niel, 2010). The results were analysed with a Galaxie Chromatography Workstation (v.1.9.3.2). The optical density of the culture was measured at 620 nm (OD_{620}) using a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). CDW was determined by filtration as previously described (Willquist & van Niel, 2010). Glucose, acetate, lactate, propionate and ethanol were analyzed by HPLC (Waters, Milford, MA, USA) on an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA) at 45°C, with 5 mM H_2SO_4 (0.6 ml·min^{-1}) as mobile phase. The column was equipped with a refractive index detector (RID-6A; Shimadzu, Kyoto, Japan). The osmolality was measured in 100µL samples using a Micro-Osmometer (Type 13DR-Autocal, Hermann Roebling Meßtechnik, Berlin, Germany).

2.4 RNA extraction and analysis

Total RNA was isolated from cells collected at steady states from chemostats performed as described above. Cells were rapidly cooled to 4˚C then harvested by

centrifugation at 6000g for 10 min at 4˚C and were immediately transferred to dry ice/ethanol broth (approximately -70˚C) prior to storage at -80˚C until further processing. Total RNA was isolated using a modified TRIzol (Life Technologies) protocol in combination with an RNAeasy kit (Qiagen). As an additional step cells were sonicated after resuspension in TRIzol reagent. cDNA was produced from the collected RNA using Superscript III reverse transcriptase (Life Technologies), random primers (Life Technologies) and the incorporation of 5-(3-amino-allyl)-2'-deoxyurinidine-5'-triphosphate (Life Technologies) as described by JCVI (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). The quality and integrity of the extracted RNA was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies).

2.5 Microarray hybridization

In order to compare the changes in transcription levels in an adapted strain with respect to medium osmolality and also with the transcription levels in WT, two three-way experimental loops were designed, one each for the strains G10 and AG6. A spotted whole genome microarray was developed for *C. saccharolyticus* using 30- to 62 – mer oligonucleotides based on its ORFs. Thus cDNA was labelled with either Cy3 or Cy5 dye (GE Healthcare) and hybridized to Corning GAPS II coated slides. Microarray slides were scanned using a Molecular Devices 400B scanner. GenePix Pro 7 was used to quantitate signal intensities, prior to analysis with JMP Genomics 5 (SAS, Cary, NC) using a mixed effects analysis of variance model. ORFs that were differentially transcribed two-fold or more and met the Bonferroni statistical criterion were considered to up- or down-regulated.

2.6 Bioinformatics analysis

All the information regarding genome sequences and corresponding annotations were retrieved from the Integrated Microbial Genomes (IMG, https://img.jgi.doe.gov/cgi-bin/w/main.cgi) (Markowitz et al., 2012).

2.7 Assessment of fitness of adapted strains

Fitness of adapted strains, *C. saccharolyticus* G10 and *C. saccharolyticus* AG6 was determined by estimating the extent of their respective growth and H_2 yield

compared to the wild-type , when all the strains were grown in similar conditions, i.e. low-osmotic condition.

2.8 Calculations

All calculations of Q_{H2} were based on the ideal gas law and the H_2 and CO_2 concentrations in the headspace and the flow rate of the flue gas, i.e. flow rate of the influent N_2 gas added to the rates of H_2 and CO_2 produced in the reactor as no other gases were detected. Product yields were calculated by determining moles of products formed per mole of glucose consumed. Biomass yield was calculated as moles of biomass formed per mole of glucose consumed. Carbon and redox balances were calculated as described previously (de Vrije et al., 2007).

3. Results

3.1 Development of osmotolerant strains of *C. saccharolyticus*

The process of evolutionary engineering was applied to develop osmotolerant strains of *C. saccharolyticus*, which involved adaptation of *C. saccharolyticus* to the increasing concentration of glucose and/or sodium acetate (Table 1 and 2). Adaptation of *C. saccharolyticus* to higher concentrations of glucose, i.e. up to 100 g/L, required more than 600 generations, which produced its variant *C. saccharolyticus* G10 capable of growing in a medium containing 100 g/L (Table 1). Similarly, more than 600 generations were required for the development of *C. saccharolyticus* AG6, which was able to grow in a medium containing higher concentrations of sodium acetate and glucose (Table 2). Both strains retained their ability to grow in high-osmotic media, even after they were grown in a low-osmotic medium (> 200 mOsmol/kg H_2O) for at least 20 generations (data not shown).

3.2 Results obtained from chemostat cultures

After confirming the stability of the adapted strains, they were cultivated in a CSTR in continuous mode in either high- or low-osmotic medium, whereas the *C. saccharolyticus* WT was cultivated only at a low-osmotic condition. Moreover, *C. saccharolyticus* G5 was cultivated in relatively high-osmotic and optimized medium

to evaluate its ability to convert higher amounts of sugars in a chemostat. The G10 strain lost its viability after about 6-7 generations entirely when grown in a chemostat with low-osmotic medium, hence a technical duplicate of steady state could not be obtained.

The G5 strain amassed about 2-fold higher amounts of biomass than all other strains regardless of the medium osmolality (Table 4). However, the WT strain displayed a significantly higher biomass yield than the rest of the strains. For the G10 strain the biomass yield and the amount of biomass were higher in a high-osmotic medium compared to that in a low-osmotic medium. On the other hand, for the AG6 strain, the biomass yield was higher in high-osmotic condition, whereas the biomass concentration was higher in low-osmotic condition.

Among the chemostats performed with low-osmotic medium, the specific substrate consumption rates q_s and specific H_2 (q_{H2}) and acetate (q_{ace}) production rates achieved by the strains G10 and AG6 were about 40% higher than those of WT. For both G10 and AG6 the values for q_s, q_{H2} and q_{ace} reduced by about 25-50% when they were grown in their respective high-osmotic media compared to those obtained in low-osmotic medium. Strain G5, consumed the substrate at a much faster rate compared to any other strains but still could not achieve the similar values for q_{H2} and q_{ace} as those obtained for G10 and AG6 when grown in low-osmotic medium. For strain G5, the specific consumption rate for xylose was at least 2-fold higher than that for glucose or arabinose.

When grown in low-osmotic medium, all strains reported nearly stoichiometric values for molar yields of H_2 and acetate. However, at high-osmotic condition, the H_2 yield was reduced by 25% for all adapted strains. The ratio of respective molar yields of H_2 and acetate for all the strains varied from 1.7-2.25 regardless of medium osmolality (estimated from Table 4) except for strain G5, for which the concerned ratio was just above 3. None of the cultures produced significant amounts of lactate or ethanol regardless of the strain or medium osmolality. The carbon and redox recovery for the strains G10, AG6 and WT generally varied between 85 and 113%. The cultivation of strain G5, however, displayed very poor carbon and redox recovery (Table 4).

Strain G5 displayed the highest volumetric H_2 productivity (Q_{H2}) i.e. approximately 21 mmol/L/h and substrate conversion rate (SCR) among all the strains regardless of the medium osmolality (Fig. 1). The WT, G10 and AG6 strains achieved similar Q_{H2} and SCR when grown in a low-osmotic medium at a substrate loading rate (SLR) of 2.5 mmol/L/h. The Q_{H2} reduced by at least 25% when G10 was subjected to higher SLR; the SCR however, remained unchanged. In contrast, for strain AG6

both the Q_{H2} and SCR reduced by more than 50% when it was grown in a high-osmotic medium. The extent of substrate conversion (SCR/SLR) was highest, i.e. about 80% for WT, G10 and AG6 strains when they were grown in a low-osmotic medium. In contrast, more than 90% of sugar was unutilized by the G10 strain, when it was grown in a high-osmotic medium.

3.3 The fitness of adapted strains

The fitness of the osmotolerant strains G10 and AG6 was determined by comparing their growth (OD and biomass concentration) and H_2 yield with that of the WT strain, when they were grown in a medium with low-osmolality (Table 4). With respect to growth both the adapted strains have lost fitness by at least 40% (Fig. 2). However, strain G10 is fitter than strain AG6. No significant differences were observed in the fitness of all the strains with respect to H_2 yields obtained by them.

3.4 Transcription analysis

To explore the changes in transcription levels of various genes within the adapted strains at either high- or low-osmotic condition compared with that of the WT, RNA samples collected at steady states were subjected to a couple of three-way microarray analysis loops. The analysis of extracted RNA with Bioanalyzer confirmed its quality and integrity sufficient for further analysis.

A single microarray experiment included three different samples – for an adapted strain (G10 or AG6) grown at i) high- or ii) low-osmotic condition, and iii) WT strain grown at low-osmotic condition, which enabled three different comparisons within an experiment.

Transcriptional analysis of the strain G10

When the transcription levels of the genes within G10 strain were compared with respect to the medium osmolality, about 176 ORFs were differentially transcribed, of which 41 encoded for hypothetical proteins. When the medium osmolality was increased the ORFs coding for hydrolytic enzymes and glycogen synthesis were down regulated, whereas the ORFs coding for sulphate assimilation and ammonia transporter were up regulated (Table. 3). When transcription levels of the genes within WT and G10 strains were compared in response to their individual growth in a low-osmotic medium, about 78 ORFs were differentially transcribed, of which

23 encoded for hypothetical proteins. Of the remaining 55 ORFs, the ORFs coding for solute/sugar transport proteins were down regulated, whereas the ORFs coding for transposition of mobile genetic elements (MGEs) were up regulated. Furthermore, when the transcription levels of genes within WT in response to a low-osmotic medium and that of strain G10 in response to high-osmotic medium were compared, about 218 ORFs were differentially transcribed, of which 59 encoded for hypothetical proteins. Of the remaining 159, the ORFs coding for solute transport related proteins, hydrolytic enzymes and glycogen synthesis were down regulated, whereas ORFs coding for proteins related to sulphate assimilation, ammonia transport and transposition of MGEs were up regulated.

Transcription analysis for the AG6 strain

When the transcription level of the genes of the AG6 strain in response to an increase in the medium osmolality was studied, about 117 ORFs were differentially transcribed, of which 32 encoded for hypothetical proteins. In addition, the ORFs coding for proteins related to glycogen synthesis hydrolytic enzymes were down regulated, whereas the ORFs responsible for sulphate assimilation and ammonia transport were up regulated (Fig. 3). When transcription levels of the genes within WT and AG6 strains were compared in response to their individual growth in a low-osmotic medium, about 207 ORFs were differentially transcribed, of which 74 encoded for hypothetical proteins. Of the remaining 133 ORFs, the proteins related to solute/sugar transport, ATP synthase complex, uracil synthesis and DNA repair were down regulated, while the ORFs coding for the proteins related to transposition of MGEs were up regulated. Moreover, when the transcription levels of genes within the WT in response to a low-osmotic medium and that of strain AG6 in response to high-osmotic medium were compared, about 242 ORFs were differentially transcribed, of which 78 encoded for hypothetical proteins. Of the remaining 164, the ORFs coding for proteins related to sugar/solute transport, ATP synthase complex, glycogen synthesis, uracil synthesis and DNA repair were down regulated, while the ORFs coding for proteins involved in sulphate assimilation, ammonia transport and transposition of MGEs were up regulated.

4. Discussion

4.1 The mechanism of adaptation in *C. saccharolyticus*

While undergoing adaptation to a stressful environment, microorganisms can respond via two principle mechanisms – i) through programmed mechanisms which mostly result in changes in phenotype without a change in genotype, and/or ii) through a change in genotype resulting in a distinct gain or loss of a phenotype (Moxon et al., 2006). However, as the natural habitat of *C. saccharolyticus* contains very low concentrations of soluble sugars and/or organic acids (Rainey et al., 1994; Willquist et al., 2010), it is likely that it does not have a mechanism required for the sensing of high-osmotic conditions (Moxon et al., 2006). On the other hand, The MGEs are known to play a pivotal role in adaptation of bacteria to a variety of stressful environments (Blot, 1994; Top & Springael, 2003). Indeed, the genes related to the transposition of MGEs were significantly up-regulated in both the adapted strains of *C. saccharolyticus* regardless of the medium osmolality, suggesting a permanent change. Therefore, as the genome of *C. saccharolyticus* contains a large number of insertion sequences that can function as MGEs (Chung et al., 2013), it increases the likelihood of *C. saccharolyticus* undergoing random mutagenesis caused by the MGEs during the adaptation to high-osmotic stress. This is further strengthened by the down-regulation of the genes related to DNA-repair in the strain AG6. However, the genomes of the adapted strains need to be studied further to confirm this hypothesis.

4.2 Clonal selection of an adapted strain and its fitness

As a prokaryote, *C. saccharolyticus* reproduces asexually (Narra & Ochman, 2006). When a population is subjected to a competition-based selection, the rate of mutations increases. However, in the microorganisms reproducing asexually different clones carrying different pools of beneficial mutations may arise, creating various subpopulations in a culture (De Visser & Rozen, 2005). This leads to a phenomenon termed 'clonal interference' where, the different beneficial mutations in different clones cannot be mixed together, thus interfering with the development of the fittest clone (Muller, 1964). On the other hand, in a step-wise adaptation process carried out in a series of batch cultures, 'clonal interference' allows selection of the fittest clone from subpopulations of clones with various degrees of fitness (Conrad et al., 2011; Elena & Lenski, 2003; Vanee et al., 2012). Since, a similar

approach was applied for the evolutionary engineering of *C. saccharolyticus* during this study, it can be argued that at the end of the process, the populations obtained consisted of only the clones of the fittest strains i.e. G10 and AG6. Moreover, the last stage of the respective evolutionary engineering experiments for the strains G10 and AG6 involved more than 200 generations which allowed for the selection of a stable mutant with most beneficial mutations.

One of the perils of evolutionary engineering via step-wise batch cultures is an occurrence of the phenomenon known as 'Müller's ratchet', which explains hitchhiking of deleterious mutations along with the beneficial mutations affecting the fitness of the resulting mutant (Elena & Lenski, 2003; Muller, 1964; Vanee et al., 2012). The fitness of an adapted strain can be defined in terms of its average reproductive success in a particular environment, typically expressed relative to its ancestral origin (Lenski & Travisano, 1994). Therefore, the adapted strains, G10 and AG6, were tested for their fitness in an environment most favourable to their ancestor, the WT strain. The loss of fitness by both the adapted strains, G10 and AG6, suggests accumulation of deleterious mutations during the process of adaptation. Moreover, the instability of strain G10 when grown in a low-osmotic medium suggests the need of a selection pressure for its maintenance. Nevertheless, the loss in fitness by the osmotolerant strains did not diminish the ability to obtain near theoretical maximum H_2 yields in a low-osmotic medium.

4.3 Fermentative performances of the strains G10 and AG6

Prior to this study, *C. saccharolyticus* was reported to be able to grow successfully in medium with no more than 150 mM of glucose (Talluri et al., 2013), which is at least 3-fold lower than the concentration of glucose the strain G10 is tolerant to. In addition, another study reported a complete inhibition to the growth of *C. saccharolyticus* in a medium containing more than 150-175 mM of sodium acetate (van Niel et al., 2003), to which the strain AG6 is tolerant to, along with 150 mM of glucose.

When the adapted strains were grown in high-osmotic medium, they appeared to invest more energy in the production of biomass. In previous studies, *C. saccharolyticus* displayed a similar phenomenon, wherein higher biomass yield were obtained when faced with a stressful wash-out situation (Pawar & van Niel, 2014; Pawar et al., 2014). In agreement with a previous study performed with the WT strain (Willquist et al., 2009), the increase in osmotic pressure did not cause a

metabolic shift to lactate and/or ethanol production in any of the adapted strains, G10, AG6 and G5. The lower carbon and electron recovery combined with the unusually lower acetate yields obtained during the cultivation using the strain G5 suggests acetyl-CoA dependent synthesis of a metabolite missing from the analysis performed. However, further analysis is needed to know whether the missing product(s) is from the recently reported inventory of novel metabolites produced by *C. saccharolyticus* when grown on various monosaccharides (Isern et al., 2013).

In a previous study, when *C. saccharolyticus* was cultivated in an UA reactor with granular sludge, it was able to withstand more than 120 mmolC6/L/h of SLR (Pawar et al., 2014). However, the maximum SCR remained well below 5 mmolC6/L/h; about 2-fold of this value was obtained during this study using strain G5. Evidently, higher SCR resulted in the highest ever Q_{H2}, i.e. 20.7 mmol/L/h reported for continuous cultures performed in CSTR using *C. saccharolyticus* (Bielen et al., 2013). Furthermore, in the present study the specific consumption rates for glucose, xylose and arabinose were significantly higher compared to those obtained in a previous study, wherein the WT strain was grown on a wheat straw hydrolysate in a sulphur-limited chemostat (Pawar et al., 2013).

4.4 Effects of adaptation on the metabolism and physiology

In a previous study, *C. saccharolyticus* was shown to assimilate sulphate efficiently, without affecting its growth and H_2 production and also that the increase in the sulphate concentration led to increase in the overall glycolytic flux (Pawar & van Niel, 2014). Similarly, when the total sulphur and nitrogen concentration in the medium was increased the strain G5 displayed higher glycolytic flux. Thus, the up-regulation of genes involved in sulphate assimilation (Pawar & van Niel, 2014) in the strains G10 and AG6 when they were grown in a medium with high amounts of glucose suggests either a sensing mechanism for availability of high concentrations of sugar or a response to sulphur limitation. Likewise, the up-regulation of the genes related to the up-take of ammonium, suggests a limitation of nitrogen, which is in agreement with the previous studies related to ammonium transporters and nitrogen-limitation in bacteria (Wirén & Merrick, 2004). Thus, these findings indicate that further optimization of the medium may improve the SCR, and consequently, the Q_{H2} further.

A recent study related to the regulation in the transcription of glycolytic genes in *Bacillus subtilis* reported constitutive expression of the glycolytic genes in response to the presence of sugar regardless of the concentration (Ludwig et al., 2001), which is

in agreement with this study. On the other hand, predictably, the genes involved in the sugar transport were down-regulated in both the adapted strains. In addition, the abundance of monomeric sugars in the medium may have prompted down-regulation of hydrolytic genes. Numerous studies have shown that excess of sugars and/or limitation of a nutrient, such as nitrogen induce glycogen synthesis in bacteria ((Preiss, 1984) and references therein), which is in contrast to the observations in this study. Finally, although the strains G10 and AG6 were adapted independently using two different solutes, the similarities in their response to the increase in the osmotic pressure indicates the genotypic and phenotypic parallelism often observed in the independently evolved populations (Hindré et al., 2012).

5. Conclusions

Evolutionary engineering is a reliable method to develop the osmotolerant strains of *C. saccharolyticus*. Thus, obtained strains displayed stable growth and H_2 production in high-osmotic medium providing an option for applying high SLR at relatively high HRT, which may improve the process economics further. Transposition of mobile genetic elements may have aided the process of adaptation regardless of the solute responsible used for the increase in the medium osmolality. The optimization of the growth medium resulted in an increase in high SCR and subsequently Q_{H2}. In addition to this, the up-regulation of the genes involved in sulphate and ammonium uptake in the adapted strains is encouraging for further optimization of the medium to enhance the SCR and the Q_{H2}. Despite these promising findings, further characterization and development of these strains at genetic level may be necessary before considering any industrial application.

Fig. 1 The volumetric H_2 productivity (Q_{H2}), the substrate loading (SLR) and conversion (SCR) rate obtained in this study. For strain G5, the sugar specific values for SCR were in mmol/L/h: glucose, 2.2±0.3, xylose, 4.44±0.05 and arabinose, 1.27±0.02; mmolC6/L/h, mmol of hexose equivalents of sugar(s) consumed/L/h.

Fig. 2 The fitness of the osmotolerant strains, G10 and AG6, in comparison with WT strain. Determined in terms of biomass density (g/L), OD @ 620 and H_2 yield (mol/mol).

6. Acknowledgement

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7. Competing interests

The authors declare that they have no competing interests.

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Table 4 Results obtained from chemostats performed under various conditions.

Parameter	Results obtained at D= 0.05 h^{-1} with various strains in media with high or low osmolality					
	G10, high	G10, low	AG6, high	AG6, low	WT low	G5, high†
Biomass conc. (g/L)	0.76±0	0.57	0.46±0.02	0.54±0	0.8±0.01	1.45±0
$q_{substrate}$ (mmol C6/g/h)	3.2±0.2	4.2	2.6±0.1	3.8±0	2.81±0.04	6.3±0.3*
$q_{acetate}$(mmol/g/h)	5.6±0	8.4	3.9±0.5	7.8±0.1	4.9±0.1	4.7±0.5
q_{H2} (mmol/g/h)	9.7±0	16.8	7.6±0.2	15.3±0.2	11±0.3	14.34±0.05
Product yield (mol/mol)						
H_2	3.1±0.2	4	2.9±0.2	4.07±0.05	3.92±0.15	3±0.2
Acetate	1.8±0.1	2	1.5±0.3	2.09±0.02	1.74±0.01	0.98±0.15
Biomass	0.78±0.05	0.65	0.62±0.03	0.54±0	1.03±0.01	0.51±0.02
Ethanol	0.04±0	0	0.03±0	0	0	0.1
Lactate	0.02±0	0	0	0	0	0.08
Carbon recovery (%)	100±7	107	85±12	113±1	103±1	75±7
Redox recovery (%)	98±7	106	85±12	112±0	100±2	68±7

*individual rates of glucose, xylose and arabinose consumption were 1.5±0.2, 3.07±0.04 and 0.88±0.02; †, this cultivation was peformed at D = 0.06 h^{-1} with a feed containing about 50 g/L of total sugars (Table 3).

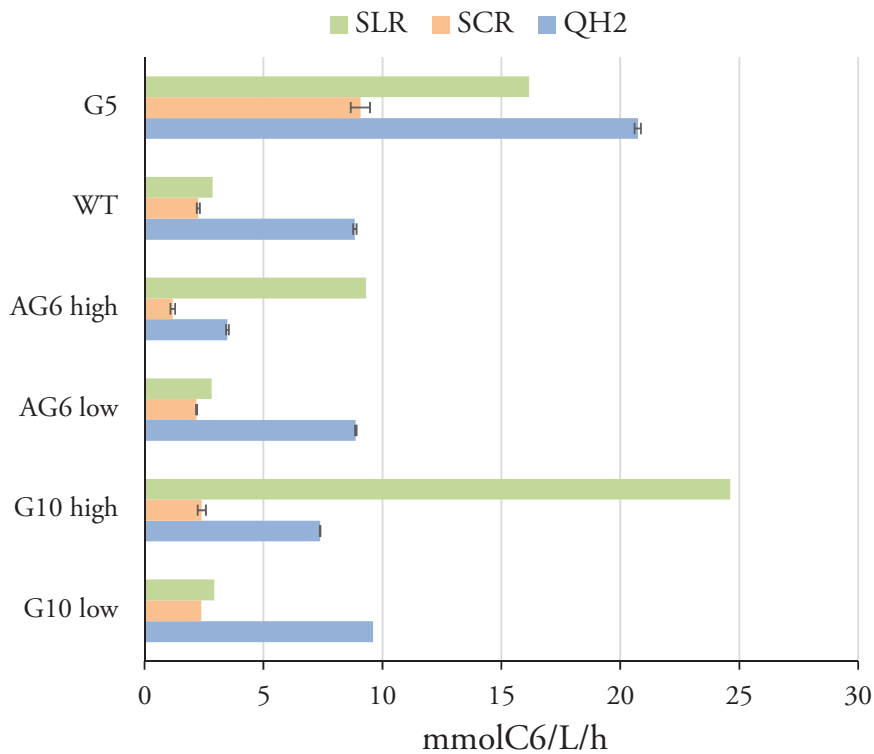
SLR
SCR
QH2
G5
WT
AG6 high
AG6 low
G10 high
G10 low
0
5
10
15
20
25
30
mmolC6/L/h

Fig. 1

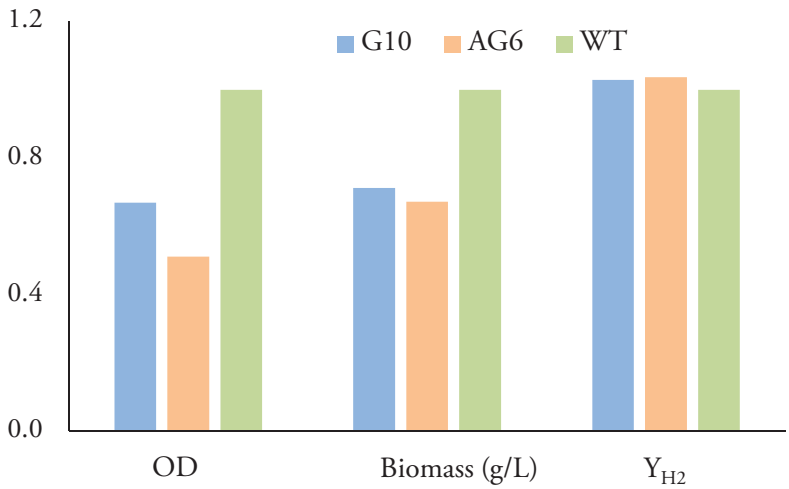
1.2
0.8
0.4
0.0
G10
AG6
WT
OD
Biomass (g/L)
Y_{H2}

Fig. 2

Figure 3 The differences between the transcriptional levels of a group of loci among the strains G10, AG6 and WT. green, lower; red, higher; and white, no significant difference

Function/phenotype	G10 high – G10 low	G10 high – WT low	G10 low – WT low	AG6 high - AG6 low	AG6 high – WT low	AG6 low – WT low
Solute transport related						
Hydrolytic enzymes						
Sulphate assimilation						
Uracil synthesis						
Ammonium transporters						
ATP synthase						
Glycogen synthesis						
Transposition						
DNA repair						
Glycolytic enzymes						

Paper VI

Genetic modification of *Caldicellulosiruptor saccharolyticus*: Elucidation of its restriction-modification system and development of a toolkit

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Abstract

Research interests pertaining to the physiology and metabolism of the cellulolytic, thermophilic, hydrogen (H_2) producer *Caldicellulosiruptor saccharolyticus* is increasing steadily over the last few years. However, unavailability of tools to perform genetic modification in *C. saccharolyticus* has impeded its development as an efficient H_2 producer. This study attempts to understand the restriction-modification (R-M) system of *C. saccharolyticus*, as well as to identify various elements and strategies to develop a vector for its genetic modification. Based on the genome sequence of *C. saccharolyticus*, it appears to possess type-I R-M system. Nevertheless, among the plasmids harboured by *Caldicellulosiruptor bescii* (pBAL and pBAS2) and *Caldicellulosiruptor kristjanssonii* (pCALKR01), only pCALKR01 could withstand the restriction endonucleases of *C. saccharolyticus*. The plasmid pCALKR01 could also resist digestion from the restriction enzyme *EaeI* (*CfrI*) suggesting that it is methylated by a cytosine-specific methyltransferase (m5C MTase). Evidently, pCALKR01 was found to contain a locus (Calkr_2643) homologous with m5C MTases, which also contains all the structural motifs present in well-known m5C MTases. Thus, methylation of a vector by m5C MTase may help overcome the R-M system of *C. saccharolyticus.* Furthermore, uracil-auxotrophic strains of *C. saccharolyticus* (*C. saccharolyticus* URA^-) were developed, with the help of 5-fluoroorotic acid (5-FOA). *C. saccharolyticus* URA^- can be used as a host for genetic modifications by using uracil-prototrophy for counter-selection as a marker. Finally, sequences for a promoter with necessary functional elements identified and a rho-independent terminator were retrieved from the genome of *C. saccharolyticus*, which can be used for designing gene cassettes of gene(s) of interest to be cloned into a vector. Thus, the knowledge gathered in this study can be used to design a battery of vectors to perform genetic modifications in *C. saccharolyticus*.

Keywords: *Caldicellulosiruptor saccharolyticus*, *C. saccharolyticus* URA^-, restriction-modification system, pCALKR01, cytosine-specific methyltransferase, promoter and rho-independent terminator

1. Introduction

Caldicellulosiruptor species are extremely thermophilic, obligately anaerobic and Gram-positive organisms possessing a natural ability to utilize various mono-, di- and, oligo-saccharides for fermentative H_2 production (Zurawski et al., 2014). Among all the well-known and well-characterised natural hydrogen-producing organisms, the members of genus *Caldicellulosiruptor* are known to possess a number of beneficial features making them the most promising H_2 producers for a commercially viable biohydrogen production process (Pawar & van Niel, 2013). *C. saccharolyticus* is one of the most advanced studied species of the genus *Caldicellulosiruptor* (Bielen et al., 2013).

However, despite years of efforts, researchers have failed to obtain a vector for genetic modification of *C. saccharolyticus*, mainly due to key bottlenecks in the research, which are, among others, – i) lack of a selection marker at the growth condition of *C. saccharolyticus* i.e. at 70°C, ii) inadequate knowledge about the restriction modification (RM) system of *C. saccharolyticus* and iii) sparse availability of native plasmids in thermophilic, Gram-positive bacteria.

The combination of enzymes with opposing activities, i.e. a restriction endonuclease (RE) and its corresponding DNA methyltransferase (MTase) constitute the R-M system in bacteria (Wilson & Murray, 1991). There are three different types of R-M systems: type I, II and III (Yuan et al., 1982). Similarly, there are three different classes of MTase: C5-cytosine-specific (m5C), N4 cytosine-specific (m4C) and N6 adenine-specific (m6A) (Wilson & Murray, 1991). The native restriction endonucleases do not cut the organisms own genomic DNA. This is due to the presence of a cognate MTase (Old & Primrose, 1981). Thus, it is essential to understand the R-M system of the host to find the compatible MTase protecting the exogenous DNA from host restriction endonucleases.

A genetic vector needs to have a selection marker to detect successful genetic transformants. The selection markers are of two different kinds – i) antibiotic marker, conferring antibiotic resistance to the host organism, and ii) nutritional (auxotrophic) markers, enabling the host organism to synthesize an essential growth factor. Since most of the antibiotics and/or heterologous enzymes conferring resistance to them are not stable at and above 70°C, antibiotic markers are not applicable in case of extreme thermophiles, such as *C. saccharolyticus* (Peteranderl et al., 1990; Wu & Welker, 1989). On the other hand, development of auxotrophic markers is mostly applicable for the organisms capable of growing in a chemically

define medium (Noll & Vargas, 1997). An auxotrophic marker was successfully developed very recently for *Caldicellulosiruptor bescii* (Chung et al., 2012). At first Chung and co-workers developed an uracil-auxotrophic strain of *C. bescii* using 5-fluoroorotic acid, and was then transformed with a vector containing a gene conferring uracil prototrophy, thus enabling the organism to grow in a defined medium (Chung et al., 2012).

In general, a site of 'origin of replication' (termed as *ori*), a protein (i.e. Rep) needed in the initiation of replication, and genes coding for other proteins aiding in replication play a significant role in the replication of the plasmid inside a host (del Solar et al., 1998). In addition to these elements, plasmids also depend upon the replication machinery of the host (del Solar et al., 1998). Thus, to develop a replicating vector for genetic modification of any organism, it is important to find native plasmids harboured by the organism(s) closely related to the desired host. Recently, Chung and co-workers developed a replicating vector for *C. bescii* and *C. hydrothermalis* using a native plasmid, pBAS2, harboured by *C. bescii* (Chung et al., 2013).

Therefore in this study, we aimed to develop the knowledge and tools essential for obtaining a vector for genetic modification of *C. saccharolyticus*. In essence, we have focused on understanding the restriction modification system of *C. saccharolyticus* and development of its uracil-auxotrophic strain to be used as a host for genetic transformation. Moreover, we have also identified a functional promoter and a terminator sequence present within the genome of *C. saccharolyticus*, to be used for the construction of a gene cassette of any gene of interest.

2. Material and methods

2.1 Microorganism and plasmids used during this study

The details of the organisms and plasmids used during this study are given in Table 1. Routine subcultures and maintenance were conducted in 250-mL serum bottles containing 50-mL of a modified DSM 640 medium (Willquist et al., 2009) unless stated otherwise. Anoxic solutions of glucose, cysteine·HCl, and magnesium sulphate were autoclaved separately and added to the sterile medium at the required concentration. A 1000X concentrated vitamins solution was prepared as described previously (Zeidan & van Niel, 2010) and used in the growth medium at 1X

concentration as a replacement to yeast extract. A 1000X concentrated trace element solution was prepared as described previously (Pawar & van Niel, 2014).

Table 1 Microorganisms and plasmids used during this study

Species	Strain	Plasmid	Reference
C. saccharolyticus	DSM 8903	-	(Sissons et al., 1987)
C. kristjanssonii	DSM 12137	pCALKR01	(Bredholt et al., 1999)
C. bescii	DSM 6725	pBAL, pBAS2	(Clausen et al., 2004)
E. coli	DH5α	pRSFDuet	Gift
E. coli	DH5α	pUC1902	Gift

2.2 Plasmid digestion with cell extracts of C. saccharolyticus

To evaluate the compatibility of the plasmids (Table 1) with *C. saccharolyticus*, plasmids were exposed to cell extracts of *C. saccharolyticus*. Firstly, *C. saccharolyticus* was cultivated in a controlled reactor with a base medium (BM) containing (per Liter): KH_2PO_4, 0.75 g; $K_2HPO_4{\cdot}2H_2O$, 1.5 g; NH_4Cl, 0.9; yeast extract, 1.0 g; resazurine, 1 mg; 1000 X modified SL-10, 1 mL, glucose, 10 g, and cysteine·HCl, 0.75 g. Solutions of glucose and cysteine·HCl were autoclaved separately and added prior to the inoculation. The reactor was set-up as described previously (Pawar & van Niel, 2014) with an exception that, reactors were continuously sparged with N_2 at 6 L/h. Cells of *C. saccharolyticus* were harvested by centrifugation at 12,000 g for 6 min. Thereafter, the cells extract was prepared as described previously (Willquist & van Niel, 2010). The endonucleases were eluted with other proteins of the cell extract and kept in a sealed anaerobic serum flask at -20°C. For the digestion experiment, 250 ng of pCALKR01, pBAL/pBAS2, and 1000 ng pRIT6 and Yip0B8, respectively, were added to 1.5 ml glass tubes with rubber stoppers with 1.5 µl of digestion buffer (Thermo Scientific, Waltham, MA, USA) and 10 µl of cell extract, under anaerobic conditions. The reaction mix was incubated overnight at 70°C. The products of reaction were run on an agarose electrophoresis gel (2% w/v) at 100 V for 1 h, in TAE buffer and DNA fragments were visualized by ethidium bromide staining and trans-illumination. Similarly, pCALKR01 and pRSFDuet were digested with *EaeI* (*CfrI*, Thermo Scientific, Waltham, MA USA) for 1 h at 37°C with recommended reaction buffer. Products of digestion were analyzed by agarose gel

electrophoresis (1% w/v) and were viewed under a gel documentation system (Molecular Imager® Gel Doc XR system, BioRad, USA).

2.3 DNA extraction

Genomic DNA of *Caldicellulosiruptor saccharolyticus* wild type and mutant strains were extracted using Easy DNA kit (Invitrogen, USA). All plasmid extractions were performed using a Qiagen Miniprep kit (#27104). All the extracted DNA samples were maintained at -20°C until further use.

2.4 Genomic analysis of the restriction-modification system in the genus Caldicellulosiruptor

The genomes of *Caldicellulosiruptor* species were analysed with the help of a web-based database - Integrated Microbial Genomes (IMG, https://img.jgi.doe.gov/cgi-bin/w/main.cgi) - to find the genes related to their respective R-M systems (Markowitz et al., 2012).

2.5 Characterization of pCALKR01

Genome of the plasmid pCALKR01 was analysed and corresponding annotations were retrieved using the NCBI nucleotide BLAST tool. GENtle software (http://gentle.magnusmanske.de) was used for DNA editing, to construct plasmid maps, restriction enzyme analysis, alignments and PCR primer design. The annotations of various coding sequences present on the plasmid were retrieved from the web-based database - Integrated Microbial Genomes (IMG, https://img.jgi.doe.gov/cgi-bin/w/main.cgi) (Markowitz et al., 2012).

2.6 Development of C. saccharolyticus URA$^-$

Three different approaches were used to obtain the uracil auxotrophic strain of *C. saccharolyticus*. Firstly, the cells of *C. saccharolyticus* were spread on a solid BM medium containing 1.75 g/L phytagel, 8 mM 5-FOA and 200 µM uracil. Plates containing 200 µM uracil but without 5-FOA were used as positive controls while plates containing neither uracil nor 5-FOA were used as negative controls. Plates were then put inside anaerobic jars and incubated at 70°C for 9 days.

Secondly, *C. saccharolyticus* was cultivated in serum flasks containing defined medium (DM) containing (per Liter): KH_2PO_4, 0.75 g; $K_2HPO_4 \cdot 2H_2O$, 1.5 g; NH_4Cl, 0.9; resazurine, 1 mg; 1000 X modified SL-10, 1 mL, vitamins solution, 1 mL, 200 µM uracil, and 8 mM 5-FOA. The preparation of anaerobic flasks was as follows: the DM medium without the carbon source, cysteine·HCl, uracil, 5-FOA and vitamins was added to the flasks and thereafter, the flasks were sealed with butyl stoppers and aluminium crimps. Similarly, serum flasks containing concentrated solutions of each of the remaining medium components were prepared. Subsequently, the headspaces of the flasks were flushed with N_2 and were autoclaved. The solutions of vitamin, uracil and 5-FOA were filter sterilized. The flasks containing a complete DM medium were then inoculated with *C. saccharolyticus* and were incubated for at least 7 days to allow the cells to adapt to the presence of 5-FOA.

Lastly, *C. saccharolyticus* was cultivated in subsequent cultivations in a DM medium without 5-FOA. One ml of each of the cultivations was transferred to a flask containing BM medium with 8 mM 5-FOA. This was performed at least three times for each sub-cultivation. Once a potential mutant was obtained, 2 ml of the cell culture was sub-cultivated into 48 ml DM medium. Nine further sub-cultivations were conducted to confirm the stability of the mutant.

To isolate the mutant from the liquid culture, the cells were grown on a BM medium with 1.75 g/L phytagel, 8 mM 5-FOA and 200 µM uracil. And again the plates were put inside anaerobic jars and incubated at 70°C for 9 days. Inside an anaerobic chamber single colonies were selected and transferred using a sterile loop into serum flasks containing 5 ml of DM medium, which were incubated for 24-48 h at 70°C. From these flasks, two further sub-cultivations were conducted in flasks with BM containing 200 µM uracil but no 5-FOA, however, these inoculations were incubated overnight (18 h).

2.7 Genotyping of C. saccharolyticus URA^-

In *C. saccharolyticus* the enzymes of uracil biosynthetic pathway are encoded by the *pyrBCFA* operon consisting of the *pyrB* (Csac_1931), *pyrC* (Csac_1932), *pyrF* (Csac_1933) and *pyrA* (Csac_1934) genes. The loss of uracil prototrophy can occur due to a deletion in the *pyrBCFA* region. *C. saccharolyticus* wild type and the uracil auxotrophic mutants 2, 3 and 4 *pyrBCFA* genes were amplified from genomic DNA using touchdown PCR with GC buffer, 0.8 mM dNTPs, and 0.2 µM forward primer pyrBCFA_F1, 0.2 µM reverse primer pyrBCFA_R1 (Table 2) and 0.08 U/µl

Phusion Hot Start polymerase (Thermo Scientific, Waltham, MA USA). The amplification protocol started with initial denaturation at 98°C for 3 min, followed by two consecutive programs – i) 10 cycles of denaturation at 98°C for 10 s, annealing at 64.4°C (-1°C/cycle until 54.4°C) for 30 s, elongation at 72°C for 3 min, and ii) 25 cycles of denaturation at 98°C for 10 s, annealing at 54.4°C for 30 sec, and elongation at 72°C for 3 min, and ended with final elongation at 72°C for 10 min. The fragment size of the amplified operon was analysed by gel electrophoresis using 1X TBE buffer and 0.8% agarose gel. Staining was performed using gel red post staining procedure.

Table 2 Primers used for amplification of *pyrBCFA* in *C. saccharolyticus*

Primer	Sequence	Product (bp)
PyrBCFA_F	CGTTGGTGGAACAAAACTCA	6716
PyrBCFA_R	AAAGAGGATAAATGCTCTCT	

2.8 Assessment of restriction modification system in C. saccharolyticus

The genomes of *C. saccharolyticus*, *C. bescii* and *C. kristjanssonii* were analysed to locate methyltransferases. The amino acid sequences of identified methyltransferases were obtained from NCBI and further analysed for conserved motifs. A multiple sequence alignment was performed using tools available in the computer software - 'BioEdit' (Ibis Bioscences, Carlsbad, CA 92008).

2.9 Identification of functional promoter and terminator sequences

A native *C. saccharolyticus* promoter region was manually selected and its functional elements were identified. The promoter is located in an intergenic region (coordinates 1967260 to 1967502) upstream to the locus Csac_1823 and probably controls the expression of housekeeping genes, such as pyruvate kinase (Csac_1831). Furthermore, the terminator sequence located in *C. saccharolyticus* was identified using Transterm software (http://mrna.otago.ac.nz/).

3. Results

3.1 Restriction modification system in C. saccharolyticus

Genomes of *C. saccharolyticus*, *C. kristjanssonii* and *C. bescii* contain the same kind of MTase, i.e. D12 class N6 adenine-specific DNA MTase (Table 3). However, these genomes differ with respect to the REs they contain (Table 3). *C. saccharolyticus* has a type-I restriction endonucleases suggesting the presence of type-I R-M system, whereas *C. bescii* and *C. kristjanssonii* have a type-II restriction endonucleases and hence type-II R-M system. Moreover, the pair of restriction endonucleases and MTase in *C. bescii* and *C. kristjanssonii* appear to be part of a single multi-subunit enzyme, as they are part of the same operon (Table 3), which is in contrast to the pair of RE and MTase in *C. saccharolyticus*.

Table 3 Genes related to R-M systems in various *Caldicellulosiruptor* species. RE, restriction endonuclease.

Organism	Locus Tag	Gene Product Name
C. bescii Z-1320, DSM 6725	Athe_2017,	D12 class m6A DNA MTase
C. bescii Z-1320, DSM 6725	Athe_2437	m4C MTase (Chung et al., 2012)
C. bescii Z-1320, DSM 6725	Athe_2438	*CbeI* (Type II RE, (Chung et al., 2012))
C. kristjanssonii DSM 12137	Calkr_2088	D12 class m6A DNA MTase
C. kristjanssonii DSM 12137	Calkr_2089	Type II RE
C. saccharolyticus DSM 8903	Csac_1403, 2595, 2752	D12 class m6A DNA MTase
C. saccharolyticus DSM 8903	Csac_2673	Type I RE
pCALKR01	Calkr_2643	m5C MTase (This study)

3.2 Compatibility of native thermophilic plasmids with C. saccharolyticus

Native plasmids pCALKR01, pBAL and pBAS2 from organisms closely related to *C. saccharolyticus* (Table 1) were isolated and subsequently incubated in duplicates with cell extract of *C. saccharolyticus*. Similarly, plasmid harboured by *Escherichia coli*, pRSFDuet and pUC1902 were also incubated in duplicates. The intact pCALKR01 plasmid depicts mainly 'open circular' and 'supercoiled' confirmations when incubated without any cell extract. In contrast, when treated with cell extract of *C. saccharolyticus* pCALKR01 mainly appears in supercoiled form suggesting digestion of 'open circular' form by the nucleases of *C. saccharolyticus*, which also explains the presence of a smear in the corresponding wells (Fig. 2). All other plasmids viz., pBAL, pBAS2, pRSFDuet and pUC1902 also displayed various stable confirmations when incubated in buffer only. However, in the presence of the cell extract, all these plasmids underwent degradation, as depicted by the smear on the electrophoresis gel (Fig. 1). Thus, among all the plasmids studied only pCALKR01 appeared to withstand restriction endonucleases of *C. saccharolyticus*.

3.3 Characterisation and annotation of pCALKR01

pCALKR01 (Fig. 3) is a low copy number circular plasmid of 15970 base pairs (bp) with GC content of 39%, which is harboured by *C. kristjanssonii* (Fig. 2). It mainly contains 17 coding sequences (CDS), of which CDS 1, 2, 5, 9, 11, 12, 14, 15 and 17 code for hypothetical proteins. Of all the remaining CDSs, the CDS 3 product is an integrase family protein; CDS 4 and 6 code for a CopG domain protein; and CDS 7 codes for a 'Helix-Hairpin-Helix glycerol proline aspartate (HhH-GPD) family protein' that is involved in nucleotide-excision repair. The CDS 10 product belongs to the 'excisionase protein family' according to the KEGG database, whereas the TIGRFAM database suggests CDS 10 to be a regulatory protein involved in mercury resistance. Interestingly, the annotation revealed that CDS 8 (i.e. Calkr_2643) codes for a C5 cytosine-specific MTase.

3.4 Characterisation of Calkr_2643

The amino acid sequence of the product of Calkr_2643 was subjected to a ClustalW analysis (multiple sequence alignment) by comparing it with amino acid sequences of the well-known m5C MTases. The amino acid sequence encoded by locus Calkr_2643, consists 5 different amino-acid sequence patterns in consensus with those of well-known m5C MTases. These consensus sequences are (amino acids

represented by their respective single-letter denotation): i) F-G-GG----F (Fig. 3A), ii) GFPC—FS-AG---G (Fig. 3B), iii) P-----ENVKN---H—G-T (Fig. 3C), iv) PQNRER-----F (Fig. 3D) and v) LYKQ-GN-V-V------A (Fig. 3E). The remainder of the MTases of any of the *Caldicellulosiruptor* species do not possess these aforementioned consensus sequences (data not shown).

3.5 Digestion of pCALKR01 with EaeI

The restriction endonuclease *EaeI* recognises and cleaves the sequence 5′-YGGCCR-3' but cannot digest a circular DNA methylated by m5C-MTases (Whitehead & Brown, 1983). Hence, to verify whether pCALKR01 is methylated by m5C-MTase, it was subjected to a digestion reaction with *EaeI*. The plasmid, pCALKR01 has two target sites for *EaeI*, which upon digestion should generate 2 fragments of approximately 12 and 4 kb in size. The plasmid pRSFDuet carries 5 target sites, and thus has the possibility of yielding 5 fragments of approx. 2.3 and 1.3 kb each, and 3 more fragments of less than 200 bp in size. The digestion reaction revealed that pRSFDuet was digested, but pCALKR01 remained intact (Figure 4).

3.6 Development of C. saccharolyticus URA$^-$

Three approaches were used to obtain uracil auxotrophic mutants of *C. saccharolyticus*: i) cultivating the cells with 5-FOA on solidified medium, ii) cultivating the cells with 5-FOA in liquid medium, and iii) pre-growing the cells with medium containing uracil but no 5-FOA and then exposing them to 5-FOA. After numerous attempts, the first two methods did not produce any mutants. However, when the cells were pre-grown on a DM medium with uracil but no 5-FOA for about 15 generations and then inoculated to the DM medium with 5-FOA, growth was observed in one of the flasks upon 12 days of incubation (data not shown). The cells from this flask were inoculated again in DM medium with 5-FOA for nine subsequent cultivations (about 30 generations) and were incubated for about 24-48 h each to ensure the stability of the phenotype, which was confirmed by a significant reduction in the lag phase. Thereafter, the mutant cells were spread on solid medium to obtain individual colonies to segregate different strains. Seven different colonies were obtained, which were then inoculated in DM medium with uracil and 5-FOA to obtain cells for further analysis and usage.

3.7 Verification of C. saccharolyticus URA$^-$

The seven different strains, termed as mutant 1 to 7, were picked from the plate and were cultivated for several generations to confirm the stable phenotype. The genomic DNA of all the mutants was extracted and the operon *pyrBCFA* was amplified. Similarly, *pyrBCFA* region from the wild-type *C. saccharolyticus* was also amplified. When the amplified products were run on the gel, it appeared that the *pyrBCFA* regions of three of the mutants were shorter than the one from the wild type (Fig. 5). However, more specific reactions are needed to pinpoint the exact locations of the deletions in the *pyrBCFA* region of the respective mutants. The identification of the correct deletion locations will help in designing the gene cassettes to be cloned into a vector as a selection marker. Due to technical difficulties these deletions were not identified at the time of drafting this manuscript.

3.8 Identification of functional promoter and terminator sequences

To construct a vector for genetic transformation of *C. saccharolyticus*, the genes coding for a marker must be cloned into a plasmid with other gene(s) of interest. To facilitate their expression these genes need a suitable promoter and also a terminator preventing synthesis of transcripts longer than required to conserve the energy. Hence, to construct a gene cassette with a sequence coding for a gene of interest, a functional promoter (Fig. 6A) and Rho-independent terminator (Fig. 6B) sequences were located within the genome of *C. saccharolyticus*. The promoter sequence consists of necessary functional elements, such as the Pribnow-Schaller box i.e. a consensus sequence of 5'-TATAAT-3', another consensus sequence, 5'-TTGACA-3', known as 'the -35 element' (Clark & Pazdernik, 2013), and the sequence 5'-ATGAGGT-3' coding for ribosomal binding site (also known as the Shine-Dalgarno sequence (Krebs et al., 2013)). The Shine-Dalgarno sequence is located in between the transcription start site and the start codon (Fig. 6A). The melting temperature of the RNA-hairpin formed by the rho-independent transcription termination sequence (Fig. 6B) found to be above 70°C, thus making it suitable for *C. saccharolyticus*.

4. Discussion

4.1 Significance of restriction modification system in bacteria

The introduction of exogenous DNA into a cell is met by many challenges, including survival of the DNA inside the cell. If the exogenous DNA is linear, it is vulnerable to degradation by the host exonucleases, whereas a circular DNA is broken down by restriction endonucleases. Chung and co-workers reported that the native restriction modification system of *C. bescii* was a main hurdle to reach successful genetic modifications (Chung et al., 2012). Possession of alternative R-M systems is not restricted to members of the same genus but can even occur between different strains of the same species (Tyurin et al., 2006). Upon evaluation of the R-M system of various members of genus *Caldicellulosiruptor* it was found that *C. saccharolyticus* and *C. bescii* have dissimilar R-M systems. This was further illustrated by the vulnerability of the plasmids of *C. bescii* to the restriction endonucleases of *C. saccharolyticus*. Hence, vectors developed for *C. bescii* and *C. hydrothermalis* (Chung et al., 2013) may not be applicable for genetic transformation of *C. saccharolyticus*.

The gene coding for a restriction endonuclease in *C. bescii*, *CbeI* (Athe_2438), is adjacent to the one coding for its cognate m4C MTase (M.*CbeI* (Athe_2437)) probably forming a multi-subunit complex. In addition to M. *CbeI*, *C. bescii* possesses a m6A MTase (Athe_2017) which is non-cognate to *CbeI*, as methylation by M.*CbeI* was found to protect a genetic vector from *CbeI*, thus allowing successful transformation of *C. bescii* (Chung et al., 2012). Similar to *C. bescii*, the restriction endonuclease and m6A MTases in *C. saccharolyticus* are not present adjacent to each other, suggesting a possible non-cognate nature of these MTases. Thus, experimental evidence is needed to confirm the protection of exogenous DNA upon its methylation by the m6A MTases of *C. saccharolyticus*.

On the other hand, based on – i) genomic analysis, i.e. homology of DNA sequence with those of known m5C MTases (Markowitz et al., 2012), ii) structural analysis, i.e. presence of all the conserved motifs found in known m5C MTases (Pósfai et al., 1989), and iii) functional analysis, i.e. inability of *EaeI* (*Cfr1*) to digest pCALKR01, it can be concluded that the locus Calkr_2643 present on pCALKR01 codes for a functional m5C MTase. Furthermore, although m5C MTase of pCALKR01 does not share any similarity with the MTases of any of the *Caldicellulosiruptor* species, it is able to protect the pCALKR01 from restriction endonucleases of *C. saccharolyticus*. Therefore, methylation of a genetic vector by m5C MTase may be critical to overcome the R-M system of *C. saccharolyticus*.

4.2 Significance of metabolic markers in extremely thermophilic organisms

The ability of *C. bescii* to be able to grow in a chemically defined medium enabled the development of its uracil auxotrophic mutant (Chung et al., 2012). Similarly, *C. saccharolyticus* is also able to grow in a chemically defined medium requiring only vitamins, salts and a carbon source for growth (Willquist & van Niel, 2012). This aided in following a similar approach as used by Chung and co-workers, i.e. to develop a uracil-auxotrophic mutant of *C. saccharolyticus* (Chung et al., 2012). The operon *pyrBCFA* consists of the genes encoding most of the enzymes responsible for synthesis of uracil in *C. saccharolyticus*. Of these genes, *pyrF* codes for orotidine 5'-phosphate decarboxylase, which converts the phosphorylated 5-FOA, i.e. 5-fluoroorotodine monophosphate to 5-fluorouridine monophosphate (5-FUP) (Boeke et al., 1984). 5-FUP is then subsequently converted to fluorodeoxyuridine by the uracil biosynthetic pathway, which is a toxic product killing the cells synthesizing uracil (Boeke et al., 1984). Thus, a deletion in any of the genes of the operon *pyrBCFA* can enable the cells to survive in a medium containing both 5-FOA and uracil. Therefore, finding the exact location of the deletion is important to design a vector for counter-selection of successful transformants. Unfortunately, the exact deletion site remains to be located in any of the phenotypically stable uracil auxotrophic mutants of *C. saccharolyticus* obtained in this study.

Alternatively, a nutritional marker that enables utilization of a specific carbon source can also be designed for selecting successful transformants. Mai and Wiegel constructed vectors containing exogenous genes coding for mannanase (pIKM3) and other cellulolytic enzymes (pIKM8) to enable and select *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 utilizing mannan and cellulose respectively (Mai & Wiegel, 2000). Interestingly, so far, no quantitative or otherwise reliable studies have reported the growth of *C. saccharolyticus* on glycerol as a sole carbon source (Bielen et al., 2013). Moreover, according to the IMG database no candidate genes appear to code for enzymes needed for assimilation of glycerol in *C. saccharolyticus* (Markowitz et al., 2012). Hence, cloning of the putative GlpFKD operon consisting all the necessary genes needed for glycerol catabolism from *Thermoanaerobacterium thermosaccharolyticum* (Tthe_0585 to 0587) can in principle be used as a metabolic marker in *C. saccharolyticus* to select the cells growing on glycerol (Markowitz et al., 2012).

4.3 Construction of vectors for genetic modification of C. saccharolyticus

The first successful study of genetic modification of any of the members of the genus *Caldicellulosiruptor* consisted of the development of a non-replicating vector (Chung et al., 2012). *C. saccharolyticus* codes for a protein essential for homologous recombination (Chen et al., 2008), i.e. RecA (Csac_2138, (Markowitz et al., 2012)). Thus, a non-replicating vector can be developed to carry out site-specific deletions in the genome of *C. saccharolyticus*.

Alternatively, a battery of replicative plasmids can be constructed using pCALKR01, pBAL or pBAS2. The plasmid pCALKR01 needs to be cloned with a gene cassette consisting of the appropriate marker, such as one conferring uracil prototrophy in between the promoter and the terminator identified during this study. In addition to a selection marker, pBAL and pBAS2 may also need to be engineered with the gene coding for m5C MTase retrieved from pCALKR01. The promoter and terminator sequences identified in this study should be compatible with the transcription and translational machinery of *C. saccharolyticus* as they are retrieved from its genome

4.4 Possible targets for genetic engineering in C. saccharolyticus

A number of modifications can be performed in the genome of *C. saccharolyticus* to improve the efficiency of hydrogen production. Under stressful conditions, *C. saccharolyticus* undergoes a metabolic shift to produce lactic acid which reduces the H_2 yield. Hence deletion of the gene coding for lactate dehydrogenase (*ldh*) may improve the H_2 yield. Indeed, the Δ*ldh* strain of *C. bescii* showed improved H_2 production by about 30% compared to the wild type (Cha et al., 2013). Alternatively, introduction of the oxidative branch of pentose phosphate pathway into the central carbon metabolism of *C. saccharolyticus* has also been proposed to increase the theoretical limit of hydrogen yield from 4 to 8 mol of H_2/mol of hexose consumed (de Vrije et al., 2007). Finally, engineering exogenous enzymes such as, glyceraldehyde-3-phosphate oxidoreductase (GAPOR) may also improve the hydrogen yield (Pawar & van Niel, 2013).

5. Conclusions

Due to the differences in their individual R-M systems the vectors developed for *C. bescii* and *C. hydrothermalis* are not applicable for genetic transformation of *C. saccharolyticus*. The plasmid harboured by *C. kristjanssonii*, pCALKR01, codes for a functional m5C MTase, i.e. performing methylation of a genetic vector, can aid in overcoming the RM of *C. saccharolyticus*. However, the uracil-auxotrophic strains of *C. saccharolyticus* obtained in this study need to be analysed further to pinpoint the precise location of the deletions. Functional gene cassettes of any of the gene(s) of interests can be prepared using the promoter and a terminator sequences identified in this study. Thus, a number of vectors can be constructed using the plasmids pCALKR01, pBAL and pBAS2 by engineering the gene cassettes of the gene(s) of interest. To sum up, the findings of this study may thus be instrumental in obtaining the 'elusive' vector for genetic modification of *C. saccharolyticus.*

Fig. 1 Graphical map of pCALKR01 displaying its coding sequences and recognition sites for *EaeI* (*CfrI*)

Fig. 2 Restriction of various plasmids by the cell extracts of *C. saccharolyticus.* *E. coli* plasmids: pRSFDuet and pUC1902, *C. bescii* plasmids: pBAL and pBAS2; -C, negative control (cell extract with buffer); and +C, positive control i.e. plasmid with reaction mixture excluding the cell extract

Fig. 3 Structural analysis of the amino acid sequence encoded by Calkr_2643 (gi 312281368). Amino acid sequences of known m5C MTases - genInfo identifier (gi) number, source organism; gi:365155231, *Bacillus smithii* 7_3_47FAA; gi:423467247, *Bacillus cereus* BAG6O-1; gi:459881844, *Helicobacter pylori* GAM270ASi; gi:163938820, *Bacillus weihenstephanensis* KBAB4; gi:429759466, *Veillonella atypica* KON; gi:313678561, *Mycoplasma bovis* PG45; gi:417939243, *Streptococcus infantis* SK970; and gi:405979196, *Actinomyces turicensis* ACS-279-V-Col4

Fig. 4. Digestion of pRSFDuet and pCALKR01 with *EaeI* (*CfrI*)

Fig. 5 Analysis of the *pyrBCFA* region of the uracil-auxotrophic mutants of *C. saccharolyticus*. W, wild type *C. saccharolyticus*; M1, M2 and M3, mutant 1, 2 and 3 respectively; NC, negative control

Fig. 6 Promoter and terminator sequence retrieved from the genome of *C. saccharolyticus*. A) Promoter sequences with its functional elements: sequences underlined with dashed lines, -10 and -35 regions; sequence underlined with solid line, Shine-Dalgarno sequence; and start codon is shown in a rectangle, B) terminator sequence

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7. Author's contribution

SSP planned the content of the article and performed some of the experiments and drafted the manuscript. AMdlH and EB performed most of the experiments under the supervision of SSP. EvN and PR were involved in the planning of the experiments, supervised the process and also critically reviewed the text. All the authors have read and approved the manuscript.

8. Competing interests

The authors declare that they have no competing interests.

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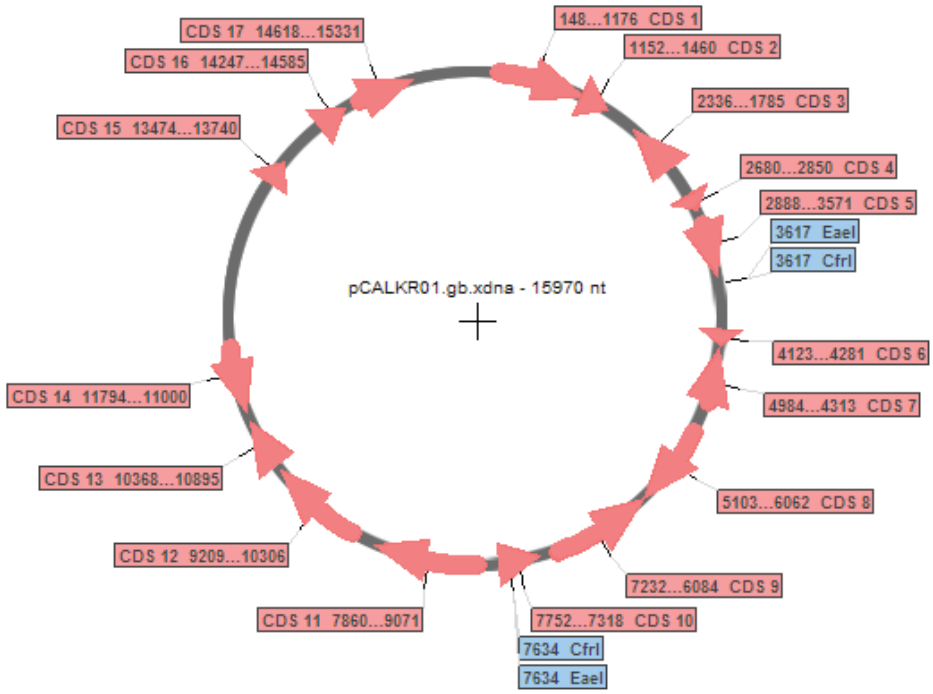
pCALKR01.gb.xdna - 15970 nt
148...1176 CDS 1
1152...1460 CDS 2
2336...1785 CDS 3
2680...2850 CDS 4
2888...3571 CDS 5
3617 EaeI
3617 CfrI
4123...4281 CDS 6
4984...4313 CDS 7
5103...6062 CDS 8
7232...6084 CDS 9
7752...7318 CDS 10
7634 CfrI
7634 EaeI
CDS 11 7860...9071
CDS 12 9209...10306
CDS 13 10368...10895
CDS 14 11794...11000
CDS 15 13474...13740
CDS 16 14247...14585
CDS 17 14618...15331

Fig. 1

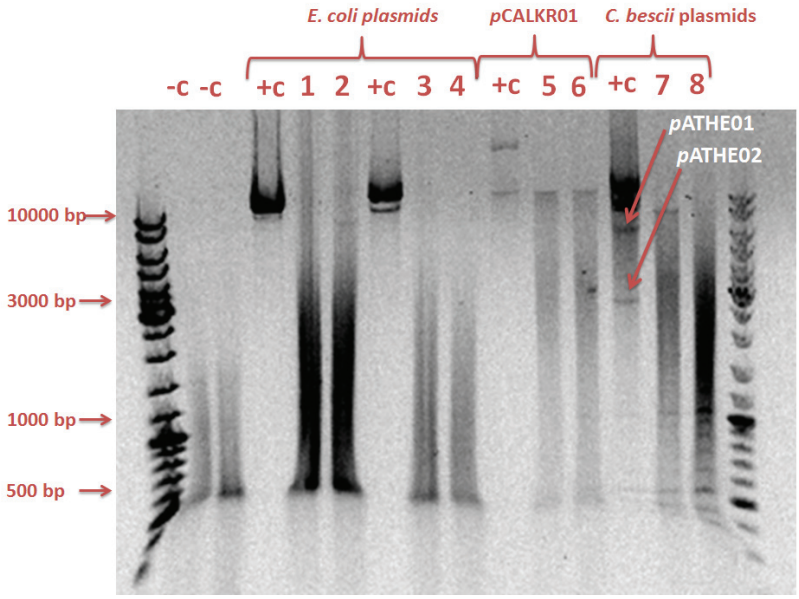
E. coli plasmids
pCALKR01
C. bescii plasmids
-c -c +c 1 2 +c 3 4 +c 5 6 +c 7 8
pATHE01
pATHE02
10000 bp
3000 bp
1000 bp
500 bp

Fig. 2

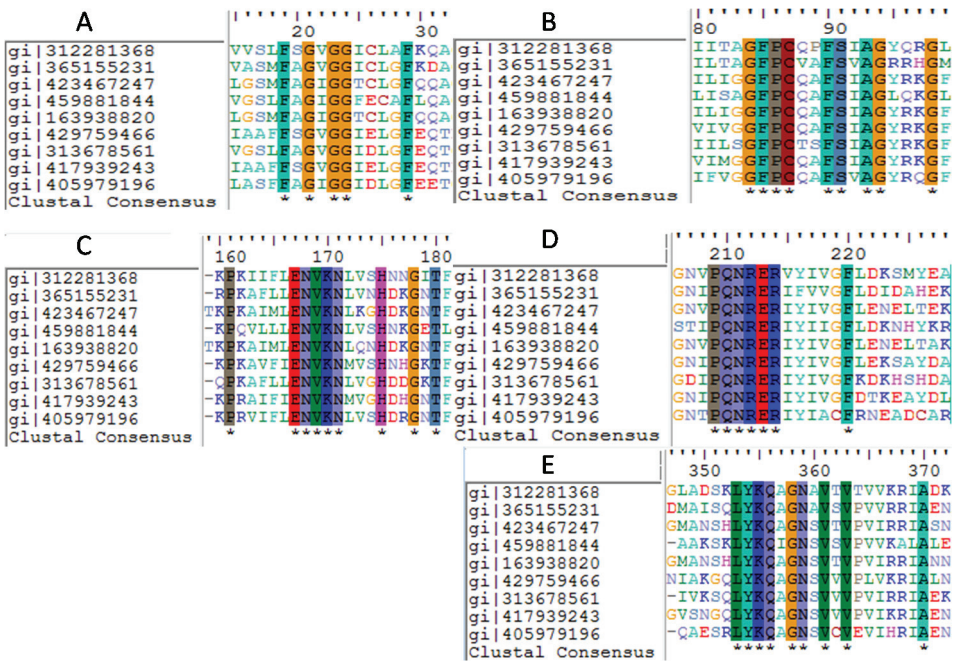
A
B
C
D
E
Clustal Consensus

Fig. 3

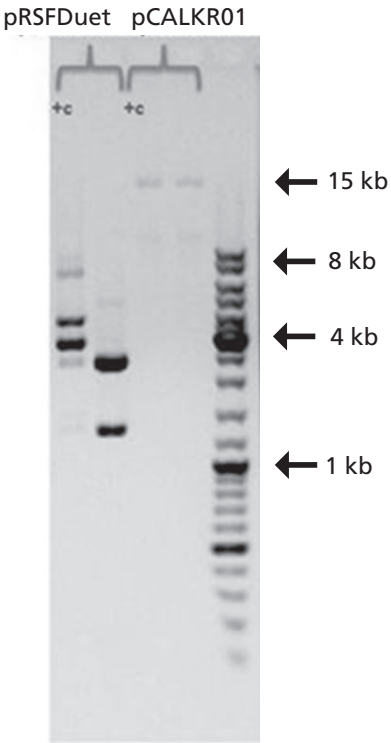
pRSFDuet
pCALKR01
+c
+c
15 kb
8 kb
4 kb
1 kb

Fig. 4

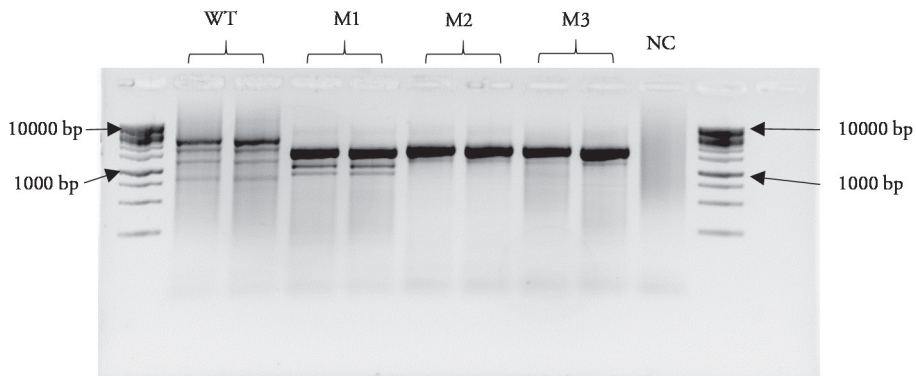
WT
M1
M2
M3
NC
10000 bp
1000 bp
10000 bp
1000 bp

Fig. 5

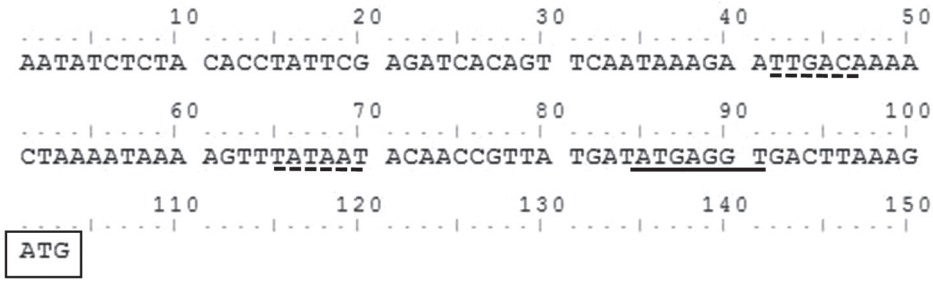
10 20 30 40 50
AATATCTCTA CACCTATTCG AGATCACAGT TCAATAAAGA ATTGACAAAA
60 70 80 90 100
CTAAAATAAA AGTTTATAAT ACAACCGTTA TGATATGAGG TGACTTAAAG
110 120 130 140 150
ATG

Fig. 6A

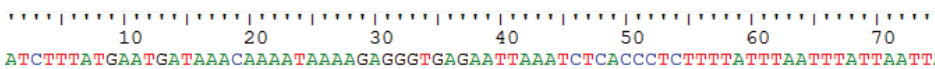
10 20 30 40 50 60 70
ATCTTTATGAATGATAAACAAAATAAAAGAGGGTGAGAATTAAATCTCACCCTCTTTTATTTAATTTATTAATTA

Fig. 6B