H synthesis from pentoses and biomass in spp.

Niels T. Eriksen, Martin Leegaard Riis, Nikolaj Kyndby Holm, Niels Iversen

Section: Biofuels and Environmental Biotechnology

H_2 synthesis from pentoses and biomass in *Thermotoga* spp.

Niels T. Eriksen*, Martin Leegaard Riis, Nikolaj Kyndby Holm, Niels Iversen

Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49, DK-9000 Aalborg, Denmark

*Corresponding author: Niels T. Eriksen

Fax: + 45 98 14 18 08

E-mail: nte@bio.aau.dk

Abstract

We have investigated H_2 production on glucose, xylose, arabinose, and glycerol in *Thermotoga maritima* and *T. neapolitana*. Both species metabolised all sugars with hydrogen yields of 2.7 – 3.8 mol mol^{-1} sugar. Both pentoses were at least comparable to glucose with respect to their qualities as substrates for hydrogen production, while glycerol was not metabolised by either species. Glycerol was also not metabolised by *T. elfii*. We also demonstrated that *T. neapolitana* can use wet oxidised wheat straws, in which most sugars are stored in glycoside polymers, for growth and efficient hydrogen production, while glucose, xylose and arabinose are consumed in parallel.

Keywords: Arabinose, biohydrogen, *Thermotoga maritima*, *Thermotoga neapolitana*, xylose

Introduction

Biohydrogen can be produced from organic materials by a range of different bacteria. Hydrogen production processes have been characterised in complex as well as in defined growth media. When glucose is used as substrate, the yield of hydrogen has for many bacterial cultures been in the range of 1-2.5 mol H_2 mol^{-1} glucose (Rachman et al. 1998, Mizuno et al. 2000, Fabiano and Perego 2002, Oh et al. 2003, Lin and Chang 2004, Morimoto et al. 2004, Kotay and Das 2007). In several hyperthermophilic bacteria belonging to the genus *Thermotoga*, yields of hydrogen on glucose is higher and may approach the theoretical maximum yield of 4 mol H_2 mol^{-1} glucose. Schröder et al. (1994) reported that *Thermotoga maritima* converted 1 mol glucose into 2 mol acetic acid and 4 mol of H_2. Takahata et al. (2001) found yields of 3.7 and 4 mol H_2 mol^{-1} glucose in *T. petrophila* and *T. naphtophila*, respectively, while van Niel et al. (2002) found a yield of 3.8 mol H_2 mol^{-1} glucose in *T. elfii*. In *T. neapolitana*, which has been extensively characterised with respect to hydrogen production, van Ooteghem et al. (2002, 2004) reported H_2 yields at or even above 4 mol H_2 mol^{-1} glucose, Eriksen et al. (2008) found 3.5-3.8 mol H_2 mol^{-1} glucose when no lactic acid was

formed, Munro et al. (2009) found 3.85 mol H_2 mol^{-1} glucose at the optimal temperature of 77^oC, and d'Ippolito et al. (2010) found 4.05 mol H_2 mol^{-1} glucose, and that 12-15% of the hydrogen was produced from yeast extract added to the growth medium. Also in other hyperthermophilic bacteria, hydrogen yields on glucose may approach the theoretical maximum (Verhaart et al. 2010).

The high yields of hydrogen in *Thermotoga* seem related to a bifurcating hydrogenase that has recently been characterised in *T. maritima* (Schut and Adams 2009). This hydrogenase needs NADH as well as reduced ferredoxin in a 1:2 stoichiometric ratio in order to function and is unable to use neither NADH nor reduced ferredoxin as the sole substrate. While the reduction potential of NADH is insufficient to effectively reduce protons to hydrogen, bacteria harbouring the bifurcating hydrogenase can use excess reducing power from reduced ferredoxin, which is a stronger reducing agent, to enhance the efficiency of proton reduction by NADH. When sugars are oxidised via glycolysis and the pentose phosphate pathway, NADH and reduced ferredoxin are produced exactly in a 1:2 stoichiometric ratio, and all reducing equivalents from sugar oxidation can therefore be used to produce hydrogen.

Most studies on hydrogen production in *Thermotoga* have used glucose as carbon source although hydrogen productions at a large scale will have to be based on cheaper substrates, such as plant biomass or waste streams like for example mash from the fermentation industry. These materials often have high contents of pentoses stored in carbohydrate polymers. Members of *Thermotoga* can grow on pentoses and encode and secrete amylases, cellulases, xylanases, pectinases and other glycoside hydrolases (Nelson et al. 1999, Chhabra et al. 2003, Conners et al. 2006). The theoretical maximal yield of hydrogen on pentoses is 3.3 mol mol^{-1}, but whether this yield can be obtained in *Thermotoga* cultures is not known.

Considerable amounts of glycerol are also available as waste from the biodiesel industry and in mash from fermentation industries where it is produced by *Saccharomyces cerevisiae*. The

genome of *T. maritima* contains coding sequences for a complete pathway for uptake and conversion of glycerol (Nelson et al. 1999), and van Ooteghem et al. (2004) found a positive signal for oxidation of glycerol by *T. neapolitana* in a microplate assay. A single report also describes hydrogen production in *T. neapolitana* on waste glycerol, although the yield of hydrogen as well as the degree of glycerol conversion was low (Ngo et al. 2009). It is therefore not clear how well *Thermotoga* is able to utilise glycerol for growth and hydrogen synthesis.

In this study we have quantified yields of hydrogen in *T. maritima* and *T. neapolitana* growing on xylose or arabinose, the two dominating pentoses in angiosperms, as well as glucose and glycerol, and demonstrated that hydrogen production in *Thermotoga* can be carried out with high yields also on complex carbohydrates.

Materials and Methods

Strain and growth media

Thermotoga maritima DSM 3109, *T. neapolitana* DSM 4359, and *T. elfii* DSM 9442 were grown in a modified version of the ATCC 1977 medium (see van Ooteghem et al. 2002) containing 1 g cysteine.HCl l^{-1} as reducing agent, and glucose, xylose, arabinose or glycerol as carbon substrate. The medium also contained 2 g yeast extract l^{-1} and 2 g peptone l^{-1}, which are needed by these bacteria. Potassium phosphate was increased from 0.6 to 6.3 g l^{-1} to increase the buffering capacity of the growth medium (Eriksen et al. 2008), while the concentrations of $MgCl.H_20$ and $CaCl_2.H_20$ were reduced to 0.2 and 0.01 g l^{-1}, respectively, to prevent formation of precipitates. MgCl, $CaCl_2$ and the carbon source (glucose, xylose, arabinose, or glycerol) were autoclaved in a separate solution before mixed with a solution containing the remaining medium constituents. A few crystals of resazurin were added as redox indicator.

T. neapolitana was also grown on media based on wheat straw, which had been pre-

treated by wet-oxidation at 195°C, under 12 bar O_2 pressure, and with 2 g Na_2CO_3 l^{-1} added for 15 min (Varga et al. 2002). Remaining solids were removed by centrifugation, while 2 g peptone l^{-1} was added.

Culture conditions

Batch cultures of *Thermotoga maritima, T. neapolitana*, and *T. elfii* were grown in 120 ml serum bottles containing 60 ml medium with shaking at 200 rpm and 80°C. Before the cultures were inoculated, the bottles were sealed by butyl rubber stoppers and flushed with N_2.

Cultures of *T. neapolitana* were also grown in a 3 l Applikon bioreactor containing 2.75 l medium. The reactor was equipped with a Pt100 temperature sensor and an autoclavable pH electrode (Mettler Toledo). Temperature and pH were automatically controlled by a heating blanket wrapped around the reactor wall, and by titration with 1 M NaOH. Cultures were stirred by a 4 bladed Rushton turbine and aerated by headspace or by sparger aeration with nitrogen gas. The exit gas was passed through a condenser at 4°C to reduce evaporation before it was passed through on-line sensors for quantification of gas-flow rate (AWM3000, Honeywell), and hydrogen (EZT3HYE 'Easy Cal' CiTiceL) and CO_2 (IRcel, City Technology, Portsmouth, UK) in the off-gas.

Sampling and analyses

Gas and liquid samples from the head-spaces of the serum bottles were taken using syringes fitted with needles. Excess gas pressure in a serum bottle was released into a syringe and the amount of gas, which had been present in the headspace, was estimated at ambient air pressure as the total volume of gas in the flask and in the syringe. H_2 and CO_2 in the headspace gas were measured by GC.: 0.3 ml headspace gas equilibrated to ambient temperature was injected at 110°C, and separated on a 2 m Hyacep Q column at 80°C. The gasses were quantified by a thermal conductivity detector

against known standards. N_2 was used as carrier at 10.3 ml min^{-1}.

Growth was determined from the OD_{800} values, and correlated to the dry weight which was determined after filtration onto pre-dried 0.2 µm disc filters and drying at 105°C, and by microscopic observation.

Glucose, xylose, arabinose, glycerol and organic acid concentrations as well as Maillard products formed in culture supernatants were quantified using HPLC: 50 µl of 0.22 µm filtered culture supernatant was separated on an Aminex HPX-87H column (Bio-rad), eluted with 0.5 mM H_2SO_4 at 0.4 ml min^{-1} and 30°C. Detection was performed with a refractive index detector. Total concentrations of glucose, xylose, arabinose in media based on wet oxidised wheat straw were measured after acid hydrolysis in 4% (w/v) H_2SO_4, for 10 min at 121°C.

Results and Discussion

Hydrogen production on glucose, xylose and arabinose

All 3 *Thermotoga* species investigated: *T. maritima*, *T. neapolitana* and *T. elfii* grew and produced hydrogen on xylose, arabinose, as well as on glucose, which was included as substrate to allow comparisons of results to previous studies. Fig. 1a shows, as an example, the consumption of arabinose and the production of hydrogen, CO_2, acetic acid and lactic acid in a 60 ml *T. maritima* batch culture grown in a closed serum bottle.

[POSITION FIGURE 1]

The stoichiometry of glucose, xylose, or arabinose conversion was quantified by plotting the produced amounts of the various metabolic products against the amount of sugar consumed (Fig. 1b). Table 1 summarises the observed molar yields of hydrogen on glucose, xylose and arabinose in *T. maritima* and in *T. neapolitana*. Two processes may have affected the yields shown in Table 1: metabolization of components from yeast extract and peptone by the cells, and by

chemical processes transforming part of the sugars into Maillard products (Fig. 1a). d'Ippolito et al. (2010) found that approx. 12-15% of the hydrogen produced in *T. neapolitana* came from conversion of such substrates, resulting in overestimation of the hydrogen yield on sugar. However, the amounts of sugar that went into Maillard reactions, predominantly in early stages of the cultures, were also in the order of 10-15%. Since the Maillard products, which were detectable by HPLC, were partly re-metabolised in late stages of batch cultures, the Maillard reactions have probably effected the estimated hydrogen yields to a lesser extend than the up-take of additional substrates, and the estimated hydrogen yields on sugars are therefore slightly higher than the true yields.

[POSITION TABLE 1]

The molar of yield of hydrogen on glucose was 3-4 in both species and similar to previously reported yields on glucose (Schröder et al. 1994, van Ooteghem et al. 2002, 2004, Eriksen et al. 2008, Munro et al. 2009, d'Ippolito et al. 2010). This indicates that our cultures have been grown at conditions that are comparable to conditions generally used for hydrogen production studies in *Thermotoga*.

The molar yields of hydrogen on xylose and arabinose were also close to or above 3. In *T. neapolitana*, the measured hydrogen yields were even above the theoretical maximal value of 3.3 mol hydrogen per mol pentose (Table 1), most likely because yeast extract and peptone have played greater roles in as substrates in *T. neapolitana* cultures compared to T. *maritima* cultures. Carbon balances, which included the sugar used as substrates, and the produced CO_2, acetic acid, and lactic acid were positive in *T. neapolitana* cultures, while slightly negative in *T. maritima* cultures. Biomass was not included in the carbon balances since the biomass is predominantly produced from components in the yeast extract and peptone.

The rate by which hydrogen was produced was highest in *T. neapolitana* cultures

(Table 1). During the growth phase, the average volumetric hydrogen production rates were 2-3 times higher in *T. neapolitana* than in *T. maritima* cultures, and similar in magnitude to the hydrogen production rates of 0.8 - 0.9 mmol l^{-1} h^{-1} previously found in *T. neapolitana* under similar conditions (Munro et al. 2009). Also the specific hydrogen production rates, estimated as the yield of hydrogen on biomass (Fig. 1b, inset) during initial phases when growth was exponential multiplied by the specific growth rate, were highest in *T. neapolitana*. Individual cells were most productive in early stages of batch cultures but gradually their activity decreased. In some cultures, activity stopped before all sugar was taken up, a phenomenon that has been explained by decreasing pH (Eriksen et al. 2008, Munro et al. 2009) but also metabolites, CO_2 and hydrogen accumulated and may have repressed the cultures. We found no systematic differences in hydrogen production rates on pentoses compared to glucose. The hydrogen yields and production rates shown in Table 1 therefore show that xylose and arabinose are at least comparable to glucose with respects to their qualities as substrates for hydrogen production in *Thermotoga*.

Glycerol as substrate for hydrogen production

Glycerol would also be an interesting substrate for hydrogen production in *Thermotoga*. However, we found no conversion of glycerol in cultures of *T. maritima*, *T. neapolitana*, nor *T. elfii* (data not shown). If glycerol was supplemented as sole carbon source, none of the 3 species grew and they did not produce more hydrogen than cultures supplemented only with yeast extract and peptone. If glycerol was supplemented simultaneously with one or more sugar species, all species took up the sugars but left the glycerol untouched.

Glycerol is apparently not a suitable substrate for hydrogen production in *Thermotoga*, possibly as a consequence of the bifurcating hydrogenase present in these bacteria. While hexoses or pentoses are metabolised with a concurrent production of NADH and reduced ferredoxin in the

1:2 stoichiometric ratio needed by the hydrogenase (Schut and Adams 2009), conversion of glycerol leads to formation of 2 mol NADH and 2 mol reduced ferredoxin per mol glycerol. Reduction of acetic acid to ethanol would be a potential route for re-oxidation of excess NADH. The *T. maritima* genome encodes a putative alcohol dehydrogenase (Nelson et al. 1999) and trace amounts of ethanol have been reported in cultures of *T. hypogea* (Fardeau et al. 1997), but ethanol was not detected in our cultures. These bacteria may therefore need access to alternative electron acceptors, like elemental sulphur (Huber et al. 1986) that can be reduced directly by NADH in order to use glycerol as substrate, but this will decrease the yield of hydrogen.

Hydrogen production on biomass based substrates

T. neapolitana grew and produced hydrogen on the liquid fraction generated from wet oxidation of wheat straw. The culture, shown in Fig 2, was grown in a bioreactor where pH was controlled and hydrogen and CO_2 were continuously removed, in order to minimise inhibition of the process and allow maximal conversion of carbohydrates. The liquid fraction used as medium contained a mixture of free xylose, glucose and arabinose (7 mM) and the same sugars bound in glycoside polymers, probably mainly hemicellulose, corresponding to a monomer concentration of 24 mM. Also 15 mM acetic acid was present after the wet oxidation.

[POSITION FIGURE 2]

After a lag of 1 day, the culture started to produce H_2 and convert the sugars while biomass concentrations increased to reach a maximum of 0.5 g l^{-1} after 2.5 days (Fig. 2a). The apparent decrease in biomass concentration after 2.5 days was at least partly associated to biofilm formation, which developed at this point and brought a fraction of the cells out of suspension. Glucose, xylose, and arabinose were taken up in parallel, as also observed in cultures grown on two or more sugars in serum bottles. de Vrije et al. (2009) also observed parallel consumption of glucose and xylose in

T. neapolitana and a second hyperthermophilic hydrogen producer, *Caldicellulosiruptor saccharolyticus* grown on *Miscanthus* hydrolysates. Most of the carbohydrates used by the culture in Fig. 2 were initially found in carbohydrate polymers, as hydrolysis was not included in the pre-treatment of the wheat straws. Glucose has previously been shown to repress β-galactosidase activity and lactose metabolism in *T. neapolitana* resulting in diauxic growth (Vargas and Noll 1996), and glucose represses the synthesis of glycoside hydrolases at the transcription level in *T. maritima* (Chhabra et al. 2003). Still, the majority of the carbohydrate polymers were degraded in periods where the total sugar monomer concentration was in the order of 5 mM (Fig. 2b). Starch in untreated potato steam peels is also a suitable substrate for hydrogen synthesis in *T. neapolitana* (Mars et al. 2010). Catabolite repression by glucose or other sugars seems therefore not to play a decisive role in the ability of *T. neapolitana* to simultaneously degrade and utilise a variety of polymeric substrates, something that is quit advantageously in relation to the employment of these bacteria in hydrogen production processes.

The culture shown in Fig. 2 consumed a total of 27 mM sugars with a corresponding hydrogen production of 72.5 mmol l^{-1}, and a hydrogen yield on sugar of 2.6 mol mol^{-1}, or 74% of the theoretical maximal value based on the relative amounts of glucose and pentoses consumed. In 9 additional *T. neapolitana* batch or fed-batch cultures grown in the bioreactor on defined media with glucose as carbon source, the hydrogen yield on glucose was 3.8 ± 0.2 mol mol^{-1} (data not shown). This corresponds to 95% of the maximum theoretical yield, and is similar to what was found in cultures grown in serum bottles (Table 1). The slightly lower molar yield of hydrogen in the culture in Fig. 2 can have been affected by the presence of alternative electron acceptors or inhibitors in the wet oxidised materials but the yield was still above what is found in mesophilic bacteria (Rachman et al. 1998, Mizuno et al. 2000, Fabiano and Perego 2002, Oh et al. 2003, Lin and Chang 2004, Morimoto et al. 2004, Kotay and Das 2007), even though culture conditions and

composition of biomass based media have not yet been optimised.

Hydrogen production potentials

Large amounts of organic feed-stocks will be needed if H_2 is to replace a considerable fraction of the hydrogen that is currently produced from fossil fuels and amounted more than 20 billion kg in the US in year 2008 (Bromaghim et al. 2010) and 3 times more world wide (van Ooteghem et al. 2002). In Table 2 we have, as an example, estimated how much of this current hydrogen production that in 2030 potentially can be replaced by bio-H_2 produced from leftover mash from the 2. generation industry. The US Department of Energy (2008) predicts a growth in bioethanol production from close to zero in 2010 to $6 \cdot 10^{10}$ kg by 2030 in the US or $1.2 \cdot 10^{11}$ kg world wide. The mash therefore represents a resource not yet utilised by other applications. Depending on feed-stock, and provided that pentose fermentation is not developed, this annual mash production will contain in the order of $2.8 \cdot 10^{11}$ or $5.6 \cdot 10^{11}$ mol pentoses in the US or world wide, respectively. From these pentoses, a maximum of $9 \cdot 10^{11}$ or $1.8 \cdot 10^{12}$ mol hydrogen can be produced to replace 9% of the current hydrogen production in the US or 6% world wide. If the hydrogen yield on pentoses is reduced to 75%, as was the case in the culture shown in Fig. 2, mash from bioethanol production will be able to provide enough feedstock to cover only 7% or 4.5% of the annual hydrogen production the US or world wide, respectively (Table 2). These numbers illustrate the massive scale by which large scale bio-H_2 needs to be produced, and the enormous challenge it will be to secure sufficient biomass resources for this purpose. This is why biological hydrogen producers should be able to utilise a broad spectrum of substrates and produce hydrogen with yields close to the theoretical maximum on hexoses as well as on pentoses. Several *Thermotoga* species fulfil both criteria, and are at the moment among the most promising candidates for employment in biohydrogen production processes.

[POSITION TABLE 2]

Acknowledgements

We thank Anne Belinda Thomsen, Risø DTU for supplying wet oxidised wheat straw.

References

Bromaghim G, Gibeault K, Serfass J, Serfass P, Wagner E (2010) Hydrogen and fuel cells: The U.S. market report. A report by the National Hydrogen Association on 2008 data.

Chhabra SR, Shockley KR, Conners SB, Scott KL, Wolfinger RD, Kelly RM (2003) Carbohydrate-induced differential gene expression patterns in the hyperthermophilic bacterium *Thermotoga maritima*. J Biol Chem 278: 7540–7552

Conners SB, Mongodin EF, Johnson MR, Montero CI, Nelson KE, Kelly RM (2006) Microbial biochemistry, physiology, and biotechnology of hyperthermophilic *Thermotoga* species. FEMS Microbiol Rev 30: 872–905

de Vrije T, Bakker RR, Budde MAW, Lai MH, Mars AE, Claassen PAM (2009) Efficient hydrogen production from the lignocellulosic energy crop *Miscanthus* by the extreme thermophilic bacteria *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Biotechnol Biofuels 2: 12

d'Ippolito G, Dipasquala L, Vella FM, Romano I, Gambacorta A, Fontana A (2010) Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. Int J Hydrogen Energy 35: 2290-2295

Eriksen NT, Nielsen TM, Iversen N (2008) Hydrogen production in anaerobic and microaerobic *Thermotoga neapolitana*. Biotechnol Lett 30: 103-109

Fabiano B, Perego P (2002) Thermodynamic study and optimization of hydrogen production by *Enterobacter aerogenes*. Int J Hydrogen Energy 27: 149-156

Fardeau M-L, Ollivier B, Patel BK, Magot M, Thomas P, Rimbault A, Rocchiccioli F, Garcia J-L (1997) *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. Int J Syst Bacteriol 47: 1013-1019

Hamelinck CN, van Hooijdonk G, Faaij APC (2005) Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass Bioen 28: 384–410

Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr YB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90^{o}C. Arch Microbiol 144: 324-333

Kotay SM, Das D (2007) Microbial hydrogen production with *Bacillus coagulans* IIT-BT S1 isolated from anaerobic sewage sludge. Biores Technol 98: 1183-1190

Lin CY, Chang R-C (2004) Fermentative hydrogen production at ambient temperature. Int J Hydrogen Energy 29: 715-720

Mars AE, Veuskens T, Budde MAW, van Doeveren PFNM, Lips SJ, Bakker RR, de Vrije T, Claassen PAM (2010) Biohydrogen production from untreated and hydrolyzed potato steam peels by the extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Int J Hydrogen Energy 35: 7730-7737

Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T (2000) Enhancement of hydrogen production from glucose by nitrogen gas sparging. Biores Technol 73: 59-65

Morimoto M, Atsuko M, Atif AAY, Ngan MA, Fakhru'l-Razi A, Iyuke SE, Bakir AM (2004) Biological production of hydrogen from glucose by natural anaerobic microflora. Int J Hydrogen Energy 29: 709-713

Munro SA, Zinder SH, Walker LP (2009) The fermentation stoichiometry if *Thermotoga neapolitana* and influence of temperature, oxygen, and pH on hydrogen production. Biotechnol Prog 25: 1035-1042

Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Nelson WE, Ketchum, KA, McDonald L, Utterback TR, Malek JA, Linher KD, Garett MM, Stewart AM, Cotton MD, Pratt MS, Phillips CA, Richardson D, Heidelberg J, Sutton GG, Fleischmann RD, Eisen JA, White O, Salzberg SL, Smith HO, Venter JC, Fraser CM (1999) Evidence for lateral gene transfer between Archae and Bacteria from genome sequence of *Thermotoga maritima*. Nature 399: 323-329

Ngo TA, Kim K-R, Nguyen T-AD, Kim M-S, Sim S-J (2009) Fermentative hydrogen production from glycerol wastes of biodiesel manufacture by *Thermotoga neapolitana*. Proceedings of the 3rd intertanional conference on fermentation technology for value added agricultural products. Khon Kaen, Thailand

Oh Y-K, Seol E-H, Kim JR, Park S (2003) Fermentative biohydrogen production by a new chemoheterotrophic bacterium *Citrobacter* sp. Y19. Int J Hydrogen Energy 28: 1353-1359

Rachman MA, Nakashimada Y, Kakizono T, Nishio N (1998) Hydrogen production with high yield and high evolution rate by self-flocculated cells of *Enterobacter aerogenes* in a packed-bed reactor. Appl Microbiol Biotechnol 49: 450-454

Schröder C, Selig M., Schönheit P (1994) Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*; involvement of the Embden-Meyerhof pathway. Arch Microbiol 161: 460-470

Schut GJ, Adams MWW (2009) The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. J Bacteriol 191: 4451-4457

Takahata Y, Nishijima M, Hoaki T, Maruyama T (2001) *Thermotoga petrophila* sp. nov. and *Thermotoga naphtophila* sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int. J. System. Evol. Microbiol. 51: 1901-1909

U. S. Department of Energy (2008) World biofuels production potential. Understanding the challenges to meeting the U.S. renewable fuel standard, 67 pp.

van Niel. EWJ, Budde MAW, de Haas GG, van der Wal FJ, Claassen PAM, Stams AJM (2002) Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellusiruptor saccharolyticus* and *Thermotoga elfii*. Int J Hydrogen Energy 27: 1391-1398

Van Ooteghem SA, Beer SK, Yue PC (2002) Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Appl Biochem Biotechnol 98-100: 177-189

Van Ooteghem SA, Jones A, van der Lelie D, Dong B, Mahajan D (2004) H_2 production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions. Biotechnol Let 26: 1223-1232

Varga E, Szengyel Z, Reczey K (2002) Chemical pretreatments of corn stover for enhancing enzymatic digestibility. Appl Biochem Biotechnol 98-100: 73-87

Vargas M, Noll KM (1996) Catabolite repression in the hyperthermophilic bacterium *Thermotoga neapolitana* is independent of cAMP. Microbiology 142: 139-144

Verhaart MRA, Bielen AAM, van der Oost J, Stams AJM, Kengen SWM (2010) Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal. Environ Technol 31: 993–1003

Legends

Fig. 1 *Thermotoga maritima* grown with arabinose as carbon substrate at stirred at 500 rpm. Initially, the headspace was flushed by 100 ml min^{-1} N_2. After 1.8 days, the N_2 was sparged into the culture at same rate. a. Concentrations of arabinose (◊), acetic acid (▲), lactic acid (Δ), and biomass (■), produced amounts of CO_2 (○) and hydrogen (●), and carbon balance (◆). b. Amounts of acetic acid (▲), lactic acid (Δ),CO_2 (○), and hydrogen (●) produced compared to the amount of consumed arabinose. Inset shows amount of hydrogen produced compared to the amount of biomass produced during initial growth phase

Fig. 2 *Thermotoga neapolitana* grown on liquid fraction of wet oxidised wheat straw. a. Amounts of hydrogen, CO_2, acetic acid (▲), and lactic acid (Δ) produced, amount of NaOH added to maintain constant pH, pH, and biomass concentration (■). b. Total concentrations of xylose (●), glucose (▼), and arabinose (◆) after acid hydrolysis, and concentrations of free xylose (○), glucose (▽), and arabinose (◊) in culture supernatant

Table 1. Observed yields of lactic acid ($Y_{HLac/sugar}$), acetic acid ($Y_{HAc/sugar}$), hydrogen ($Y_{H2/sugar}$) and CO_2 ($Y_{CO2/sugar}$), carbon balance, average volumetric rate of hydrogen production (r_{H2}) during growth phase, specific rate of hydrogen production (q_{H2}) during initial exponential growth phase of batch cultures of *Thermotoga maritima* and *T. neapolitana* grown with glucose, xylose or arabinose as carbon source. Number of replicate cultures indicated by n.

Species		Unit	Carbon substrate		
			Glucose	Xylose	Arabinose
T. maritima	$Y_{HLac/sugar}$	mol mol^{-1}	0.1 ± 0.0	0.1 ± 0.0	0.2±0.1
	$Y_{HAc/sugar}$	mol mol^{-1}	1.3 ± 0.0	1.2 ± 0.0	1.3±0.1
	$Y_{H2/sugar}$	mol mol^{-1}	3.2 ± 0.1	2.7 ± 0.0	3.2±0.3
	$Y_{CO2/sugar}$	mol mol^{-1}	1.4 ± 0.1	1.3 ± 0.1	1.6±0.2
	C-balance	% per day*	-1.6 ± 0.1	-2.6 ± 0.1	-0.6 ± 1.5

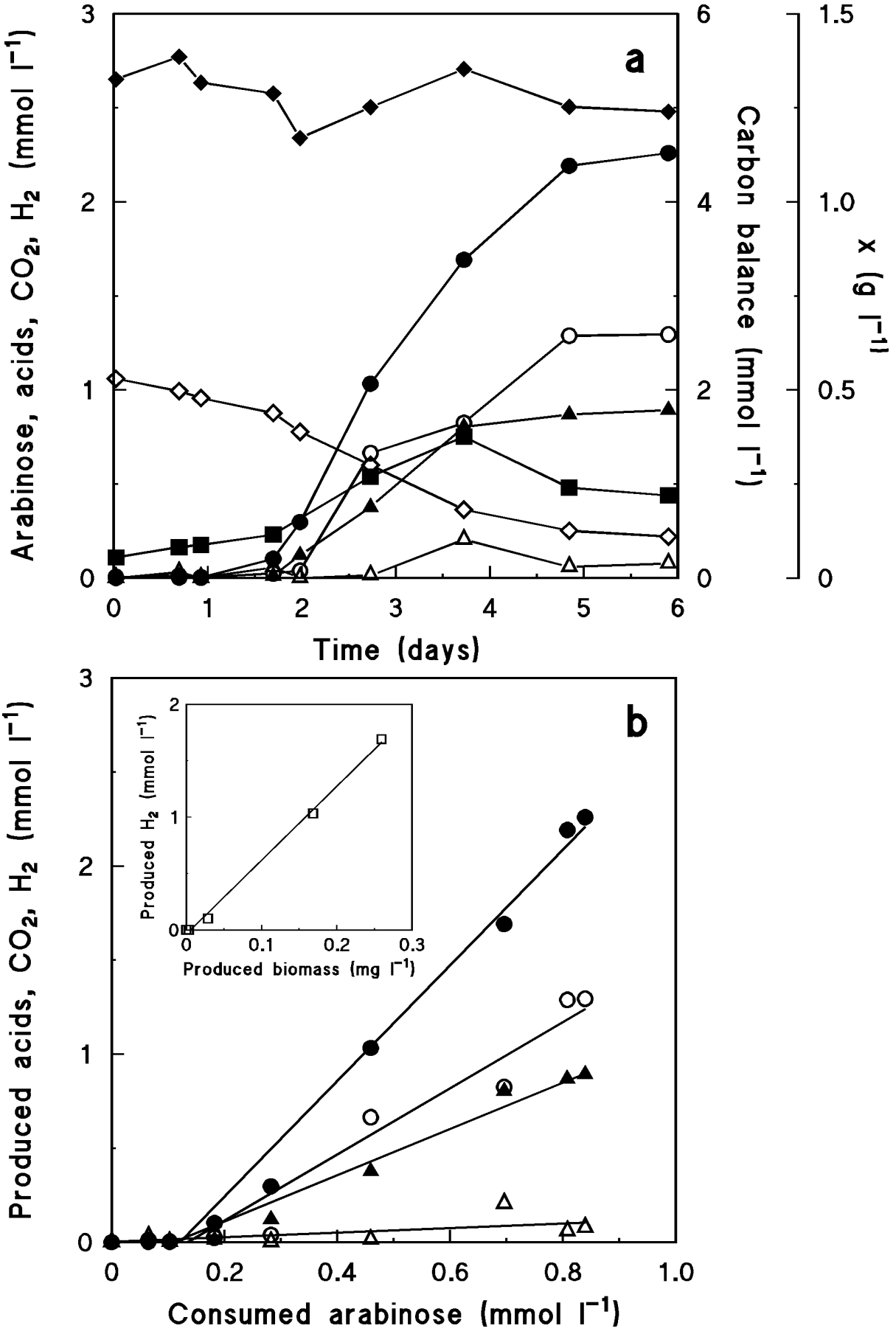
	r_{H2}	mmol l^{-1} h^{-1}	0.61 ± 0.03	0.42 ± 0.05	0.59 ± 0.07
	q_{H2}	mol g^{-1} day^{-1}	0.10 ± 0.02	0.04 ± 0.02	0.05 ± 0.01
	n		2	2	3
T. neapolitana	$Y_{HLac/sugar}$	mol mol^{-1}	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
	$Y_{HAc/sugar}$	mol mol^{-1}	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
	$Y_{H2/sugar}$	mol mol^{-1}	3.8 ± 0.4	3.4 ± 0.3	3.8 ± 0.5
	$Y_{CO2/sugar}$	mol mol^{-1}	2.4 ± 0.3	2.9 ± 0.3	2.4 ± 0.5
	C-balance	% per day*	0.5 ± 0.1	8.9 ± 2.4	4.4 ± 1.8
	r_{H2}	mmol l^{-1} h^{-1}	1.24 ± 0.09	1.45 ± 0.13	0.97 ± 0.04
	q_{H2}	mmol g^{-1} h^{-1}	0.21 ± 0.02	0.24 ± 0.04	0.24 ± 0.02
	n		6	4	2

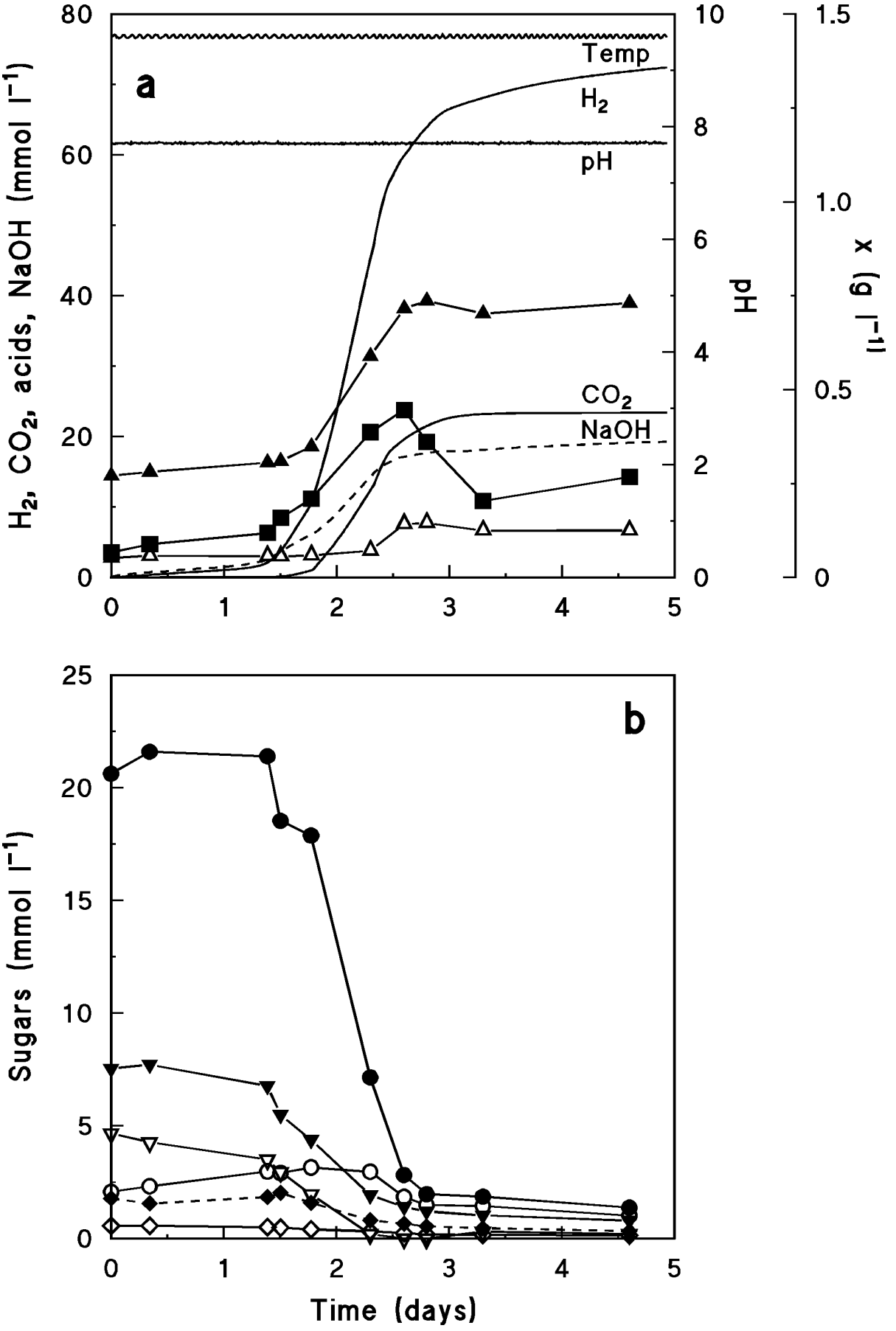
*Carbon balance is expressed as % change in total carbon content per day until stationary phase relative to initial carbon content.

Table 2. Potential biohydrogen production and replacement of fossil fuel based hydrogen production from conversion of leftover pentoses (C_5) in mash from 2. generation bioethanol production in the US and world wide in year 2030. Estimate is based on hydrogen production in 2008.

Premises	Unit	Quantity	
		USA	World
Mass H_2 production in 2008	kg $year^{-1}$	$>2 \cdot 10^{10}$, [a]	$6 \cdot 10^{10}$, [b]
Molar H_2 production in 2008	mol $year^{-1}$	$>10^{13}$	$3 \cdot 10^{13}$
Mass production of 2. generation bioethanol in 2030	kg $year^{-1}$	$6 \cdot 10^{10}$, [c]	$1{,}2 \cdot 10^{11}$, [c]
Molar production of 2. generation bioethanol in 2030	mol $year^{-1}$	$1.4 \cdot 10^{12}$	$2.8 \cdot 10^{12}$
Consumption of glucose for ethanol, glucose → 2 ethanol	mol $year^{-1}$	$7 \cdot 10^{11}$	$1.4 \cdot 10^{12}$
Generation of pentoses in mash, C_5:C_6 = 0.4 in feedstock [d]	mol $year^{-1}$	$2.8 \cdot 10^{11}$	$5.6 \cdot 10^{11}$
Potential H_2 production from C_5, $Y_{H2/pentose}$ = 3.3 mol mol^{-1}	mol $year^{-1}$	$9 \cdot 10^{11}$	$1.8 \cdot 10^{12}$
Potential H_2 production from C_5, $Y_{H2/pentose}$ = 2.6 mol mol^{-1}	mol $year^{-1}$	$7 \cdot 10^{11}$	$1.4 \cdot 10^{12}$
Potential H_2 replacement in 2030, $Y_{H2/pentose}$ = 3.3 mol mol^{-1}	%	9	6
Potential H_2 replacement in 2030, $Y_{H2/pentose}$ = 2.6 mol mol^{-1}	%	7	4.5

[a]Data from Bromaghim et al. (2010)
[b]van Ooteghem et al. (2002) estimated 3 times H_2 production world wide compared to the US
[c]Calculated from data published by US Department of Energy (2008)
[d]Predicted from data in Hamelinck et al. (2005)


a
Arabinose, acids, CO_2, H_2 (mmol l^{-1})
Carbon balance (mmol l^{-1})
x (g l^{-1})
Time (days)
b
Produced acids, CO_2, H_2 (mmol l^{-1})
Consumed arabinose (mmol l^{-1})
Produced H_2 (mmol l^{-1})
Produced biomass (mg l^{-1})


a
Temp
H2
pH
CO2
NaOH
H2, CO2, acids, NaOH (mmol l-1)
pH
x (g l-1)
b
Sugars (mmol l-1)
Time (days)