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Glycolytic pathway and hydrogen yield studies of the extreme thermophile *Caldicellulosiruptor saccharolyticus*

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Abstract NMR analysis of ^{13}C-labelling patterns showed that the Embden–Meyerhof (EM) pathway is the main route for glycolysis in the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Glucose fermentation via the EM pathway to acetate results in a theoretical yield of 4 mol of hydrogen and 2 mol of acetate per mole of glucose. Previously, approximately 70% of the theoretical maximum hydrogen yield has been reached in batch fermentations. In this study, hydrogen and acetate yields have been determined at different dilution rates during continuous cultivation. The yields were dependent on the growth rate. The highest hydrogen yields of 82 to 90% of theoretical maximum (3.3 to 3.6 mol H_2 per mol glucose) were obtained at low growth rates when a relatively larger part of the consumed glucose is used for maintenance. The hydrogen productivity showed the opposite effect. Both the specific and the volumetric hydrogen production rates were highest at the higher growth rates, reaching values of respectively 30 mmol g^{-1} h^{-1} and 20 mmol l^{-1} h^{-1}. An industrial process for biohydrogen production will require a bioreactor design, which enables an optimal mix of high productivity and high yield.

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Introduction

Hydrogen is rapidly becoming a much desired vector for clean energy. In view of the quest for sustainable energy economies, it is however of prime importance to produce hydrogen from renewable primary energy sources and not from fossil reserves.

The production of hydrogen from biomass is one of the realistic options to establish a diverse portfolio of renewable hydrogen production technologies. Besides, interest for thermochemical technologies, fermentative hydrogen production by employing bacteria to convert biomass to hydrogen is increasingly gaining attention (Nandi and Sengupta 1998; Benemann 1996; Claassen et al. 1999; Levin et al. 2004; Hagen 2006).

Several studies have already shown the ability of various micro-organisms to produce hydrogen from carbohydrates or sugars (de Vrije and Claassen 2003). Amongst the types of hydrogen-producing micro-organisms, distinct differences have been observed. For example, the hydrogen yields of thermophilic bacteria and Archaea, growing at temperatures above 60°C, often show higher values as compared to those of mesophilic bacteria growing at ambient temperatures (reviewed by de Vrije and Claassen 2003; Hallenbeck 2005). This is mainly due to the formation of different end products of the fermentation. Many mesophilic bacteria yield more reduced end products like lactate, ethanol, butanol and butyrate in contrast to thermophilic bacteria, which usually produce acetate as the main product of the fermentation.

The hydrogen yields theoretically also depend on the catabolic route of hexose conversion to intermediary C_3-compounds like pyruvate and glyceraldehyde-3-phosphate, as higher hydrogen yields are possible when the pentose phosphate (PP) pathway is used instead of the Embden–

Meyerhof (EM) and Entner–Doudoroff (ED) pathways. Studies on glycolytic pathways in (hyper)thermophilic micro-organisms have been done for saccharolytic Archaea, like *Pyrococcus furiosus*, showing the presence of modified (EM) and (ED) pathways (Kengen et al. 1996; Ronimus and Morgan 2003; Verhees et al. 2003). The sugar-degrading pathways in (hyper)thermophilic hydrogen-producing bacteria have been less extensively studied (Ronimus and Morgan 2003), with the exception of the hyperthermophile *Thermotoga maritima* (Schröder et al. 1994; Schönheit and Schäfer 1995; Selig et al. 1997). *T. maritima* ferments glucose via both the conventional EM and ED pathways.

One of the thermophilic bacteria that showed high hydrogen yields on mono- and disaccharides is *Caldicellulosiruptor saccharolyticus* (van Niel et al. 2002; Kádár et al. 2004). Until now, studies were restricted to cultures growing in batch mode. Generally, in batch fermentations, conditions are continuously changing due to different growth phases, decreasing substrate and increasing product concentrations. For *C. saccharolyticus*, it has been reported that hydrogen production and growth is inhibited by product formation, i.e. by high hydrogen pressures (above 10–20 kPa) and high acetate concentrations (due to high ionic strength; van Niel et al. 2003). This study presents results of fermentations in a continuous mode under steady state conditions on the relation of hydrogen productivity and yield to growth of *C. saccharolyticus*. Besides, the metabolic route for glucose metabolism in *C. saccharolyticus* has been analysed by ^{13}C-labelling experiments to determine the maximum theoretical yield of hydrogen on glucose.

Materials and methods

Microorganism and medium

C. saccharolyticus DSM 8903 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. *C. saccharolyticus* was grown in DSM 640 medium without trypticase. Medium was sterilised at 121°C for 20 min and made anaerobic by flushing with N_2. Sterile, anaerobic solutions of glucose, $MgCl_2$ and cysteine–HCl were added separately after sterilisation.

Continuous cultivation

Cultures to be used for inoculation were grown at 70°C in 250-ml anaerobic serum bottles with 60 ml of culture medium with 4 g/l of glucose (pH 7.0). Batch and continuous cultivations were done in a jacketed 2-l bioreactor (Applikon, Delft, The Netherlands) at a working volume of 0.75 l and a growth temperature of 72.5±0.5°C. The pH was controlled at 6.7 (measured at room temperature) by automatic addition of 2 N NaOH. Cultures were continuously stirred at 350 rpm and sparged with N_2 at 7 l h^{-1}. Medium reservoirs were flushed with nitrogen.

Batch fermentations were inoculated with 10% (v/v) of cultures in the exponential growth phase. After 8 or 18 h, continuous cultivations were started. For this, two experimental setups were used. Medium containing 4.4 g/l glucose and 1 g/l yeast extract (purchased from Duchefa, Haarlem, The Netherlands) was added to the bioreactor in steps of increasing dilution rates up to D=0.35 h^{-1}. In other experiments, glucose concentrations (approximately 2 or 4 g/l) and dilution rates (approximately 0.1 or 0.3 h^{-1}) were alternately changed. A culture was considered to have reached steady state when the hydrogen production rates remained constant (<3% variation), and at least five volume changes had occurred. Samples of 10 ml were regularly taken for determination of optical density of the culture and analysis of substrate and products by high-performance liquid chromatography (HPLC). Besides, samples of 100 ml were collected at steady states for the determination of cell dry weight (CDW) and dilution rate. Hydrogen and CO_2 were regularly measured in the off gas.

^{13}C-labelling experiments

C. saccharolyticus was cultured in anaerobic, sterile bicarbonate-buffered medium consisting of (per liter) KH_2PO_4 of 0.41 g, K_2HPO_4 of 0.64 g, $MgCl_2.6H_2O$ of 0.4 g, $NaHCO_3$ of 2.8 g and NH_4HCO_3 of 1.3 g. Yeast extract, trace elements, $FeCl_3$ and cysteine were present in the same concentrations as in DSM 640 medium. Fermentation of ^{13}C-glucose occurred in 118-ml anaerobic serum bottles with 10 ml culture medium under a nitrogen/CO_2 atmosphere (80/20%) with 4 g/l ^{13}C-glucose labeled at the C1-, C2- or C3-atom (isotopic enrichment minimal 99%; Campro Scientific, Veenendaal, The Netherlands). Fermentations were started with 2% (v/v) inoculum derived from a 30-ml batch culture that was in the exponential growth phase and from continuous cultures at dilution rates of 0.09 and 0.29 h^{-1}. Incubation of the bottles was at 70 or 72°C for 31 to 50 h. Gas samples were regularly taken from the headspace for determination of H_2 production by gas chromatography (GC). At the end of the fermentation, supernatant of the cultures was collected by centrifugation and stored at −20°C for further analyses by HPLC and NMR.

NMR experiments

The supernatants of batch fermentations with labeled glucose were freeze dried and subsequently dissolved in

0.5 ml 99.9% D_2O (Isotec, USA). ^1H- and ^{13}C-NMR spectra were measured at a probe temperature of 300 K on a Bruker AMX-500 spectrometer (Bruker, USA). Eight scans with a repetition time of 2.3 s and 2,000 scans with a repetition time of 1.5 s were recorded for the ^1H- and ^{13}C-spectra, respectively. The identification of the observed resonances in the ^{13}C-NMR spectra was done by spiking with pure compounds.

Biomass analysis

For CDW determination, 100 ml of fermentation medium was centrifuged (8,000×*g*, 15 min), and the pellet was washed with 80 mM NaCl in 14 mM potassium phosphate buffer (pH 7.0). The pellet was resuspended in deionized water and dried at 70°C until constant weight. The optical density of the culture was measured against a water blanc at 580 nm after dilution of the culture broth with deionized water on a Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The relation between OD and CDW was found to be $CDW[g/l]=(0.377\times OD_{580}+0.011)$ ($R^2=0.985$). A thermogravimetric analyser (TGA 7; Perkin-Elmer, The Netherlands) was used to determine the ash content of cell pellets. For this, approximately 30 mg of dry matter was heated from 25 to 900°C with a rate of 20°C/min. The mean ash content was 2.8% (±0.5%) of the total CDW.

The elemental composition of *C. saccharolyticus* that was grown in a continuous glucose-limited culture at a dilution rate of 0.1 h^{-1} was determined. For this, cell pellets were prepared as described for CDW determinations, except that the pellets were washed with deionized water. The molar ratio, calculated from the C, H, N, S and P analyses after correction for water and ash content, was found to be $CH_{1.62}O_{0.46}N_{0.23}S_{0.0052}P_{0.0071}$, which corresponds to a molecular weight of 24.6 g per mol C and a degree of reduction (γ) of 4.08 mol e^- per mol C.

Analytical methods

The concentration of glucose and organic acids in the culture supernatant was analysed by HPLC using a Shodex ionpak KC811 column (Waters, The Netherlands) at 80°C, with 3 mM H_2SO_4 as the mobile phase (1 ml/min) followed by detection by differential refractometry. Ammonium was determined with the LCK 303 test kit of Hach Lange (Düsseldorf, Germany). Hydrogen in the headspace of serum bottles was measured by GC using a RVS MolSieve 5A, 60/80 mesh, 3 m×1/8″ column. The temperature of the thermal conductivity detector, injector and column was 100, 80 and 50°C, respectively. N_2 was used as the carrier gas. Hydrogen and CO_2 production in bioreactors were monitored at regular intervals on a CP 4900 micro GC equipped with a thermal conductivity detector (Varian, The Netherlands). Hydrogen was measured on a MolSieve $MSA^{HI}BF$ module, and the temperatures of the injector and column were 60 and 80°C, respectively. CO_2 was monitored using a Pora Plot Q, $PPQ^{HI}BF$ module, and the temperature of the injector and column were both 80°C.

Calculation of carbon and redox balances

Carbon balances at steady states were calculated from the molar fluxes of the products (organic acids, CO_2 and biomass; in mmol C per h) divided by the molar flux of the consumed substrate (glucose; in mmol C per h). The redox balances were calculated in a similar way by multiplying the molar fluxes with the corresponding degree of reduction in the products and substrate (γ, in mol electrons per C-mol) including hydrogen as a product (molar flux times 2 mol of electrons per 1 mole of hydrogen).

Results

Metabolic routes

Labelling studies with ^{13}C-glucose labelled on the C1-, C2- or C3-atom were performed to discriminate between the EM, ED and PP pathways. Besides H_2 and CO_2, acetate is known to be the main end product of the anaerobic fermentation of glucose by *C. saccharolyticus*, whereas lactate can also be produced, as well as trace amounts of ethanol (Rainey et al. 1994). Depending on the position of the labelled C-atom in glucose and the glycolytic pathway that is being employed, acetate and lactate become either labelled at different C-atoms or the label ends up in CO_2/bicarbonate (see Table 1 for labelling patterns).

Growing batch cultures of *C. saccharolyticus* produced approximately 20 mM acetate after incubation with 25 mM labelled glucose. Very little lactate was produced, probably because the maximum partial H_2 pressure in the gas phase was less than 10 kPa, which is below the concentration (10–20 kPa) causing a metabolic shift from acetate to lactate production (van Niel et al. 2003). The results of the labelling studies at 72°C with an inoculum derived from a continuous culture growing at a dilution rate of 0.09 h^{-1} are shown in Fig. 1 (^{13}C-NMR spectra). Fermentation of [1-^{13}C]glucose resulted in isotopic enrichment of the methyl carbon of acetate (24.2 ppm; Fig. 1a), indicative for glycolysis via an EM-type pathway. With [2-^{13}C]glucose, the label was mainly incorporated into the carboxyl group of acetate (182.3 ppm; Fig. 1b), which is the expected position for both EM- and ED-type pathways. No enriched acetate was found after fermentation of [3-^{13}C]glucose (Fig. 1c), which would be formed when the ED or

Table 1 Labelling patterns of products after metabolism of ^{13}C-labelled C1-, C2- and C3-glucose to acetate and lactate via three glycolytic pathways

Pathways	Product	Substrate		
		[1-^{13}C]Glucose	[2-^{13}C]Glucose	[3-^{13}C]Glucose
Embden–Meyerhof	Acetate[a]	C1	C2	CO_2/HCO_3^-
	Lactate[b]	C1	C2	C3
Entner–Doudoroff	Acetate	CO_2/HCO_3^-	C2	C1
	Lactate	C3	C2	C1
Pentose–phosphate	Acetate	CO_2/HCO_3^-	C1	C2
	Lactate	CO_2/HCO_3^-	C1	C2

[a] Acetate, [1]CH_3–[2]COO^-;
[b] Lactate, [1]CH_3–[2]CHOH–[3]COO^-

PP pathway was employed. When the EM pathway is used, the label should end up in CO_2/HCO_3^-. Indeed, a low intensity resonance that was corresponding to bicarbonate (161.0 ppm) was visible on enlarged spectra, which was absent in the spectra of the samples that were obtained with [1-^{13}C] and [2-^{13}C]glucose. Because of its volatility, most of the labelled bicarbonate is likely to be lost during freeze drying of the culture supernatant before NMR analysis. The enlarged spectra showed several other enriched products that were present in low amounts. In the samples of the fermentations with [1-^{13}C] and [2-^{13}C]glucose, enriched lactate could be identified (21.0 and 69.5 ppm, respectively), which is again indicative for glycolysis via the EM pathway. Other resonances that could be identified originated from amino acids (results not shown).

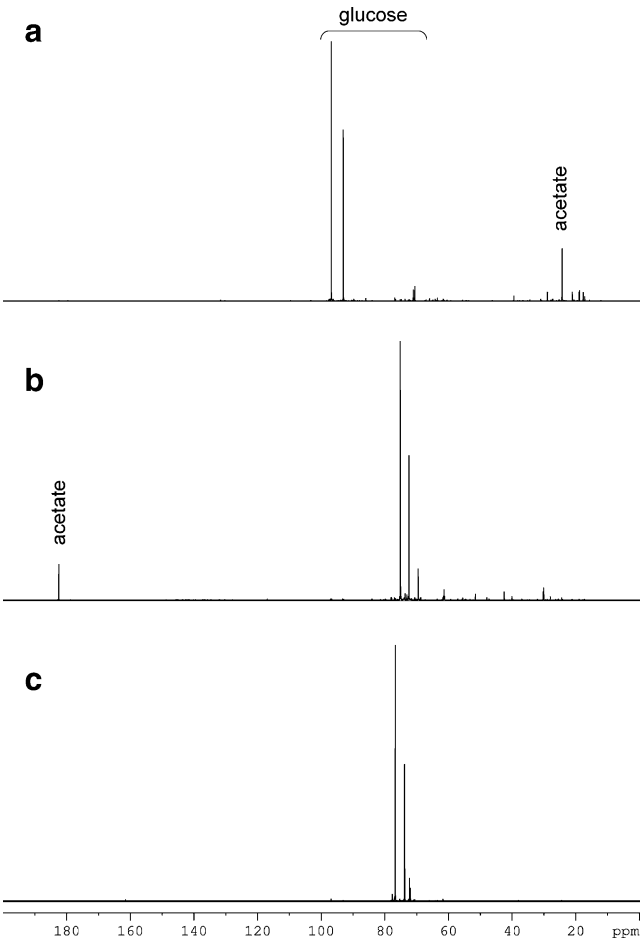

Fig. 1 ^{13}C-NMR spectra of supernatants of *C. saccharolyticus* cultures grown on labelled glucose as substrate for 31 h at 72°C. **a** [1-^{13}C]glucose; **b** [2-^{13}C]glucose; **c** [3-^{13}C]glucose. The inoculum originated from a continuous culture growing at a dilution rate of 0.09 h^{-1}. The strong intensity resonances in the 71–97 ppm region are from labelled glucose

None of the enriched products are indicative for glycolysis via an ED- or PP-pathway, except for acetate that was enriched at the carboxyl group from labelling with [2-^{13}C]glucose. However, the latter enrichment is also expected to be formed by the EM pathway. Experiments were repeated with other inocula and growth conditions. The labelling patterns were found to be independent of the origin of the inoculum (either from batch cultures or from continuous cultures growing at dilution rates of 0.09 and 0.29 h^{-1}) or the growth temperature (70 or 72°C).

^{1}H-NMR allows to quantify the relative contribution of the various glycolytic pathways of glucose to acetate metabolism (Schäfer et al. 1994; Selig et al. 1997). The ^{1}H-spectra showed the resonance patterns due to the methyl groups of acetate (Fig. 2). The central resonances at 1.91 ppm are due to the unlabelled methyl group of acetate, and the lateral resonances, separated by approximately 0.26 ppm, are due to the ^{13}C-labelled methyl groups. The percentage of enriched acetate can be calculated from the integration of the resonances of the methyl groups of acetate after correction for the 1.1% natural abundance of ^{13}C and the presence of unlabelled acetate derived from the inoculum (<3% of produced acetate). In addition, only half of the acetate molecules will become labelled from glucose that was labelled on one C-atom. These calculations showed that >99% of the acetate was produced by metabolism of glucose via an EM-type pathway (99.1%± 0.0, n=2; Fig. 2a). The percentages of enriched acetate as a product of glycolysis via the PP- or ED-pathway were very low, 3.1%±1.5 and 1.4%±0.2, and less accurate (Fig. 2b and c, respectively). The results clearly show that an EM-type pathway is the main or only glycolytic pathway in *C. saccharolyticus*.

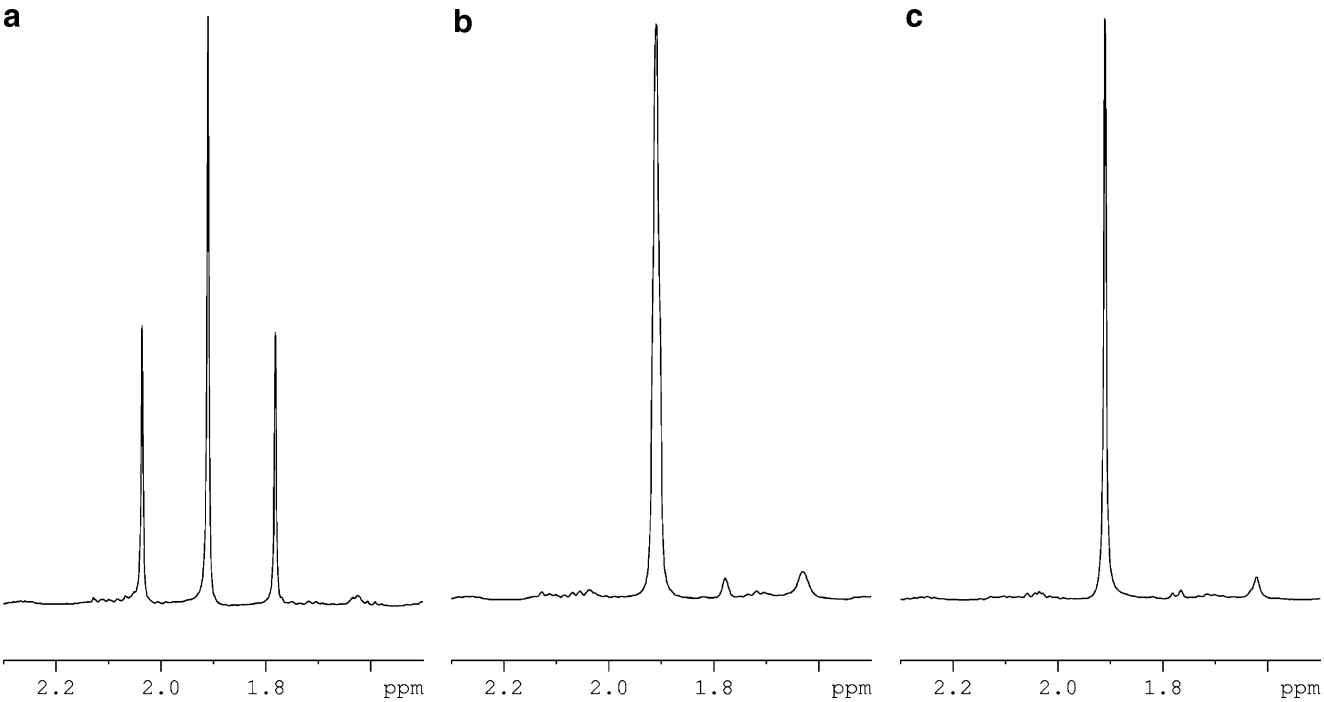

Fig. 2 ^{1}H-NMR spectra of supernatants of *C. saccharolyticus* cultures grown on labelled glucose as substrate for 31 h at 72°C. Only the resonances of acetate, the main product of the fermentation, are shown. **a** [1-^{13}C]glucose; **b** [2-^{13}C]glucose; **c** [3-^{13}C]glucose

Effect of growth rate on hydrogen yield and productivity

The effect of the growth rate on substrate consumption and product formation by *C. saccharolyticus* was studied during continuous cultivations. For this, *C. saccharolyticus* was grown on 24 mM (4.4 g l^{-1}) glucose and the dilution rate (D) of the culture was stepwise increased from 0.05 to 0.35 h^{-1}. At each steady state, when the growth rate equals the dilution rate, the concentrations of glucose and the main products in the liquid effluent were measured (Tables 2, 3 and 4). No residual glucose was detected in the culture effluent at low dilution rates, but the consumption of glucose became incomplete at dilution rates of 0.15 h^{-1} and higher. Initially, the biomass concentration increased with higher dilution rates up to 0.1 h^{-1} but decreased when glucose started to accumulate, suggesting that other factors than glucose became limiting at higher dilution rates. Acetate was the only fermentation product that was detectable in the liquid effluent. No lactate or other products were found. H_2 and CO_2 were present in the gas effluent besides the stripping gas N_2. The mean carbon recovery of the continuous culture was 0.93±0.03, which confirmed that acetate, CO_2 and biomass were the main carbon-containing products of the fermentation. The mean redox balance was 0.96±0.03, indicating accurate H_2 measurements. At D=0.4 h^{-1}, the optical density of the culture rapidly decreased, and the experiment was stopped.

The specific rates (q) of the consumption of glucose and production of hydrogen, acetate and CO_2 all increased with increasing dilution rates (Tables 2, 3 and 4). The maximum q_{H_2} and volumetric productivity (Q_{H_2}) of 30 mmol g^{-1} h^{-1} and 12 mmol l^{-1} h^{-1}, respectively, were obtained at a

Table 2 Fermentation details of a continuous culture of *C. saccharolyticus* on glucose (4.4 g l^{-1} or 24.6 mM) at steady states of different dilution rates (yeast extract, 1 g l^{-1}; pH 6.7; 72°C)

Dilution rate h^{-1}	Liquid effluent			Gas effluent		Carbon balance	Redox balance
	Biomass (g CDW l^{-1})	Glucose (mM)	Acetate (mM)	H_2 %	CO_2 %		
0.05	0.45	0.0	41.9	1.1	0.5	0.94	0.97
0.10	0.61	0.0	39.5	2.3	1.0	0.95	1.00
0.15	0.58	3.3	30.0	2.5	1.1	0.89	0.92
0.20	0.45	8.9	20.4	2.4	1.0	0.90	0.93
0.30	0.41	12.4	15.2	2.9	1.2	0.93	0.96
0.35	0.42	12.7	15.1	3.2	1.6	0.96	0.97

CDW Cell dry weight in grams per litre

Table 3 Fermentation details of a continuous culture of *C. saccharolyticus* on glucose (4.4 g l^{-1} or 24.6 mM) at steady states of different dilution rates (yeast extract, 1 g l^{-1}; pH 6.7; 72°C)

Q_{H_2} Volumetric hydrogen productivity in mole H_2 per litre per hour

Dilution rate h^{-1}	Specific consumption/production rate (mmol g^{-1} h^{-1})				Q_{H_2} (mmol l^{-1} h^{-1})
	$q_{glucose}$	q_{H_2}	$q_{acetate}$	q_{CO_2}	
0.05	2.8	9.3	4.7	4.3	4.2
0.10	4.1	14.5	6.5	6.3	8.9
0.15	5.6	16.4	7.8	7.4	9.5
0.20	6.9	20.2	9.0	8.8	9.1
0.30	8.8	27.0	11.1	11.2	11.0
0.35	9.9	29.8	12.7	15.0	12.4

growth rate of 0.35 h^{-1}. The specific consumption and production rates were linearly related to the growth rate (μ; being equal to D at steady state) according to the relationship formulated by Luedeking and Piret (1959), in which $q = \alpha\mu + \beta$, with α (in mmol g^{-1}) and β (in mmol g^{-1} h^{-1}) as the growth-associated and non-growth associated constants, respectively (Fig. 3):

$$\begin{aligned} q_{H_2} &= 66.6D + 6.7 \quad r^2 = 0.993 \\ q_{CO_2} &= 32.4D + 2.6 \quad r^2 = 0.962 \\ q_{acetate} &= 25.5D + 3.7 \quad r^2 = 0.994 \\ q_{glucose} &= 23.5D + 1.8 \quad r^2 = 0.993 \end{aligned}$$

According to these equations, product formation is both growth- and non-growth related.

At each dilution rate, the molar yields of the products (mol product per mol consumed glucose) were calculated (Tables 2, 3 and 4) and appeared to be higher at lower dilution rates. Consistent with this are the molar yields that were calculated from the non-growth associated constants (β), resulting in molar yields of 3.7 mol H_2 and 2.1 mol acetate per mol glucose. These values approximate the maximum theoretical yields of 4 mol H_2 and 2 mol acetate per mol glucose metabolized via the EM pathway.

The growth yield (Y_{xs}) in grams of dry matter per mol of utilized glucose increased with increasing dilution rates, which can be attributed to the use of a relative larger part of the substrate for maintenance at low growth rates. The maintenance coefficient m_s and the theoretical maximum growth yield Y_{XS}^{max} are usually calculated from the relation formulated by Pirt (1965): $\frac{1}{Y_{XS}} = \frac{m_S}{\mu} + \frac{1}{Y_{XS}^{max}}$. However, these parameters cannot be reliably derived from our results because the limiting nutrient for growth varied as growth became glucose-sufficient at higher dilution rates.

The biomass yield Y_{ATP} (gram CDW per mol ATP) was based on the assumption that 2 mol of ATP are produced per mole of acetate according to the conventional EM pathway for glucose metabolism. Y_{ATP} increased with higher dilution rates from 5.3 to 13.8 g mol^{-1}. Similar to Y_{xs}, Y_{ATP} was lower at low dilution rates due to the relatively larger part of ATP that was used for maintenance at low growth rates. For many anaerobic fermentative bacteria, an average value of 10.5 g of CDW per mol ATP is found (Bauchop and Elsden 1960). Similar values for *C. saccharolyticus* were obtained at moderate growth rates of 0.15–0.2 h^{-1}, indicating that, indeed, 2 mol of ATP per mole of acetate was formed. Previously, a ratio of 1 mol of ATP per mole acetate was reported for *C. saccharolyticus* growing in batch culture on sucrose (van Niel et al. 2002). However, the ATP yield on acetate might have been underestimated due to the low μ_{max} of 0.07 h^{-1} of this culture.

In subsequent continuous cultivation experiments, glucose concentrations of 1.9 and 4.1 g l^{-1} were applied in the

Table 4 Fermentation details of a continuous culture of *C. saccharolyticus* on glucose (4.4 g l^{-1} or 24.6 mM) at steady states of different dilution rates (yeast extract, 1 g l^{-1}; pH 6.7; 72°C)

Dilution rate h^{-1}	Molar yield (mol mol^{-1})			Y_{xs} (g mol^{-1})	Y_{ATP} (g mol^{-1})
	Y_{H_2}	$Y_{acetate}$	Y_{CO_2}		
0.05	3.3	1.7	1.6	18.0	5.3
0.10	3.6	1.6	1.5	24.6	7.7
0.15	2.9	1.4	1.3	26.9	9.7
0.20	2.9	1.3	1.3	29.1	11.1
0.30	3.1	1.3	1.3	34.1	13.5
0.35	3.0	1.3	1.5	35.4	13.8

Y Molar yield in moles of product per mole of consumed glucose, which was calculated by $q_{product}/q_{glucose}$. Y_{xs} Growth yield in grams of cell dry weight per mole of consumed glucose. Y_{ATP} biomass yield in grams of cell dry weight per mole of ATP produced. ATP production is calculated according to: $concentration_{ATP} = 2\ concentration_{acetate}$

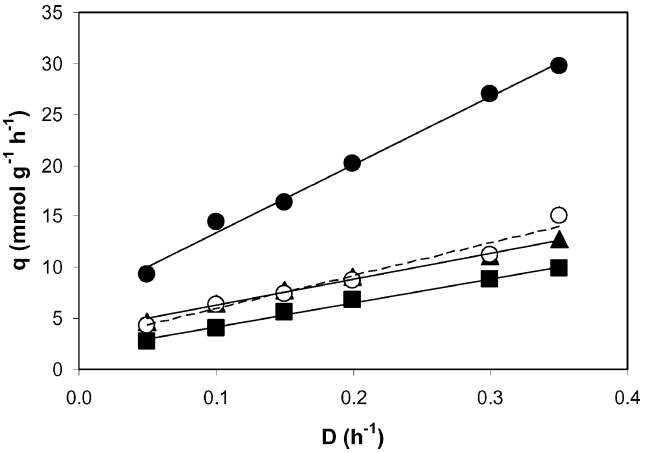

Fig. 3 Effect of dilution rate on the specific production rates of H_2 (*filled circle*), CO_2 (*open circle*, *dotted line*) and acetate (*filled triangle*), and the specific consumption rate of glucose (*filled square*) of a continuous culture of *C. saccharolyticus* on glucose (4.4 g/l) at steady states of different dilution rates (pH 6.7; 72°C)

feed at dilution rates of 0.1 and 0.3 h^{-1} (Tables 5 and 6). As the concentration of yeast extract remained at 1 g l^{-1}, the yeast extract to glucose ratio increased from 0.24 to 0.53 g g^{-1} at the lower glucose concentration. With 1.9 g l^{-1} and D=0.09 h^{-1}, hardly any glucose was detectable in the effluent, whereas at D=0.3 h^{-1} about 5% of the glucose was not consumed. The carbon balance at D=0.09 h^{-1} was 1.11, which was higher than the theoretical maximum. This might be caused by the use of yeast extract for biomass production, which is not included in the carbon balance, although it has been shown that *C. saccharolyticus* does not grow with yeast extract as the sole carbon- and energy source (van Niel et al. 2002). Furthermore, with 4.1 g l^{-1} of glucose, hardly any glucose was detected in the steady state culture at D=0.1 h^{-1}. However, the growth yield on glucose (Y_{xs}) became 20% lower than the Y_{xs} obtained with 1.9 g l^{-1} of glucose at D=0.09 h^{-1}, whereas Y_{ATP} remained similar. These data suggest that more biomass is produced from glucose instead of yeast extract at a lower yeast extract to glucose ratio.

The molar yields of hydrogen and acetate were higher at D=0.1 h^{-1} compared to the yields at D=0.3 h^{-1}, which was consistent with the results found in the first experiment (Tables 2, 3, 4, 5 and 6). The value for the specific hydrogen production rate (q_{H_2}) was independent of the glucose concentration in the feed, but the volumetric hydrogen productivities (Q_{H_2}) at the same dilution rates were less with 1.9 g l^{-1} of glucose due to the lower cell density.

Glucose started to accumulate in the culture medium at D>0.1 h^{-1} with a glucose concentration of 4 g l^{-1} (Tables 2, 3, 4, 5 and 6), indicating that growth of *C. saccharolyticus* became limited by other medium component(s) than glucose. Ammonium was present in excess, as approximately only 10% of the amount that was present in the feed was used at D=0.3 h^{-1} (results not shown). To determine whether yeast extract was the growth limiting factor, the yeast extract to glucose ratio was further increased to 1.0 g g^{-1} during continuous cultivations at D=0.3 h^{-1} with 4.0 g l^{-1} glucose. In these cultures, glucose consumption increased from 59 to 92%, and the culture density became nearly two times higher. Accordingly, the volumetric hydrogen productivity (Q_{H_2}) increased to a value of 20 mmol l^{-1} h^{-1}, whereas Y_{H_2} and q_{H_2} were not significantly affected. The results showed that components of yeast extract were limiting during glucose sufficient growth of *C. saccharolyticus*. Studies on the nature of these components will be subject for further research. No detectable amounts of lactate were measured in any of the tested conditions of continuous culturing.

A μ_{max} of 0.07 h^{-1} has been reported for *C. saccharolyticus* growing in batch culture with sucrose as the substrate (van Niel et al. 2002). In this study, *C. saccharolyticus* reached steady states at much higher dilution rates, i.e. growth rates, on glucose. The μ_{max} of *C. saccharolyticus* cells originating from cultures that were growing at steady state at dilution rates of 0.1 and 0.3 h^{-1} and from flask cultures derived from glycerol stocks were determined during pH-controlled batch cultivations by monitoring the increase in the culture density over time. Besides, the μ_{max} was determined during wash-out experiments after continuous culturing. *C. saccharolyticus* that had been growing at a dilution rate of 0.1 h^{-1} had a μ_{max} of approximately 0.2 h^{-1}, which was comparable to the μ_{max} of a batch culture originating from a glycerol stock

Table 5 Fermentation data of continuous cultures of *C. saccharolyticus* at two different glucose concentrations (1.9 and 4.1 g l^{-1}) at steady states of two different dilution rates (yeast extract, 1 g l^{-1}; pH 6.7; 72°C)

Glucose in feed [g l^{-1} (mM)]	D (h^{-1})	Glucose in effluent (mM)	Glucose consumption (mM)	C-balance	Y_{xs} [g CDW (mol Glc)$^{-1}$]	Biomass (g CDW l^{-1})
1.9 (10.7)	0.09	0.1±0.1	10.6±0.6	1.11±0.02	28.3±1.5	0.30±0.02
	0.30	0.6±0.7	10.1±0.2	0.99±0.05	38.8±4.8	0.39±0.05
4.1 (23.0)	0.10	0.1±0.1	23.0±1.3	0.96±0.02	23.1±1.0	0.53±0.05
	0.30	10.5±1.4	11.9±1.0	0.95±0.03	36.3±2.2	0.44±0.05

Values are the mean of four experiments±standard deviation.

Table 6 Fermentation data of continuous cultures of *C. saccharolyticus* at two different glucose concentrations (1.9 and 4.1 g l^{-1}) at steady states of two different dilution rates (yeast extract, 1 g l^{-1}; pH 6.7; 72°C)

Glucose in feed [g l^{-1} (mM)]	D (h^{-1})	Molar yield [mol (mol Glc)$^{-1}$]		Y_{ATP} (g CDW mol ATP^{-1})	H_2 productivity	
		Y_{H_2}	$Y_{acetate}$		q_{H_2} (mmolg^{-1} h^{-1})	Q_{H_2}(mmol l^{-1} h^{-1})
1.9 (10.7)	0.09	4.0±0.1	1.8±0.0	7.7±0.4	13.3±1.2	4.0±0.3
	0.30	3.3±0.1	1.4±0.1	13.7±2.5	25.6±5.1	9.9±0.5
4.1 (23.0)	0.10	3.5±0.1	1.6±0.0	7.1±0.5	14.5±0.3	7.7±0.8
	0.30	3.1±0.1	1.4±0.1	13.5±0.6	26.5±2.9	11.6±0.9

Values are the mean of four experiments ± standard deviation.

(Table 7). However, our previous continuous cultivations already showed that *C. saccharolyticus* can grow at higher dilution rates, as steady states were obtained at D=0.3 and 0.35 h^{-1} (Tables 2, 3, 4, 5 and 6). Wash-out experiments of cultures grown at D=0.3 h^{-1} resulted in a μ_{max} of 0.5 h^{-1}, which suggests that *C. saccharolyticus* is able to increase its maximal growth rate. In addition, batch cultures that were inoculated with a steady state culture at D=0.3 h^{-1} had a significantly higher growth rate than 0.2 h^{-1}. Preliminary experiments even suggested that steady states can be reached at a dilution rate of 0.7 h^{-1}. However, in this culture, the glucose consumption and biomass production were very low, but hydrogen production was stable.

Discussion

The ^{13}C-labelling experiments in this study show that an EM-type pathway is the main route for glycolysis in the extreme thermophile *C. saccharolyticus*. The identification of the specific type of EM pathway, which could be the conventional type or a modified EM as is common in several hyperthermophilic archaea, awaits further detailed studies of intermediates of glucose catabolism and measurements of enzyme activities. No evidence has been found for the presence of the ED or PP pathway in *C. saccharolyticus*. Analysis of the draft genome sequence showed that *C. saccharolyticus* is a Clostridial-type bacterium (http://www.img.jgi.doe.gov). Most of the genes that are encoding for the enzymes of the conventional EM pathway are present (automatic annotation by the DOE/Joint Genome Institute). Genes that are encoding for enzymes involved in the oxidative part of the PP pathway and for 2-keto-3-deoxy-6-phosphogluconate aldolase, a key enzyme of the ED pathway, have not been found. Both findings support the results of this study.

Table 7 Maximal specific growth rates (μ_{max}) of *C. saccharolyticus* (pH 6.7; 72°C)

Origin of inoculum/culture	Maximal specific growth rate, μ_{max} (h^{-1})[a]	
	Batch	Wash-out
Glycerol stock	0.19±0.04	n.a.
Steady state at D=0.1 h^{-1}	0.23±0.03	0.19±0.02
Steady state at D=0.3 h^{-1}	0.38±0.05	0.52±0.07

[a] μ_{max} was determined in a batch culture (inoculum derived from batch culture of a glycerol stock or from steady states of continuous cultures) and in a wash-out experiment. In the latter situation, the D was raised to 0.3 and 0.9 h^{-1} after steady states at D=0.1 and 0.3 h^{-1}, respectively. *n.a.* Not applicable. Values are the mean of two or three experiments±standard deviation.

In contrast, enzymatic studies and NMR analysis of ^{13}C-labelling patterns showed that both the classical EM and ED pathways are used by *Thermotoga maritima* in a ratio of 85 to 15 (Schröder et al. 1994; Selig et al. 1997). Although both micro-organisms are hydrogen-producing thermophilic bacteria, their positions in the evolutionary tree are different. *T. maritima* belongs to the order Thermotogales and represents the most ancestral sugar-fermenting organism of the bacteria. Almost one quarter of its genome shows similarity with that of the Archaea (Nelson et al. 1999).

Glucose fermentation via the conventional EM pathway results in theoretical yields of hydrogen, acetate and ATP of 4, 2 and 4 mol per mole glucose, respectively. Previous studies with *C. saccharolyticus* reported hydrogen yields of 2.6, 2.2 and 5.9 mol H_2 per mol glucose, xylose and sucrose, which corresponds to 63, 67 and 74% of the theoretical maximum, respectively (Kádár et al. 2004; van Niel et al. 2002). These data were derived from cultures growing in batch mode where conditions are continuously changing. To eliminate these effects, product yields and related growth parameters of *C. saccharolyticus* have been determined at various steady states during continuous cultivation. The hydrogen and acetate yields were dependent on the growth rate of *C. saccharolyticus*, with the highest yields being obtained at low growth rates. Values of 3.3 to 3.6 mol of hydrogen per mole of glucose have been measured, corresponding to 82 to 90% of the theoretical maximum. At growth rates higher than 0.15 h^{-1}, the hydrogen yield dropped to 2.9 to 3.1 moles of hydrogen per mole of glucose, i.e. 72 to 78% of the theoretical

maximum. Apparently, the highest yields were obtained when a relatively larger part of the consumed glucose is used for maintenance, i.e. at low growth rates.

Although the hydrogen yields that were obtained with *C. saccharolyticus* are the highest that have been reported for anaerobic fermentations at this scale, higher hydrogen yields are theoretically possible by fermentation of glucose via the PP pathway. In this case, glucose is metabolized to glyceraldehyde 3-phosphate and fructose 6-phosphate. Glyceraldehyde 3-phosphate is subsequently converted to pyruvate and acetate, and fructose 6-phosphate is used for further processing of new glucose molecules. In theory, 8 mol of hydrogen per mole of glucose will be produced, together with 2 mol of ATP and 1 mol of acetate. Presently, no thermophilic organisms are known to use the PP pathway in this way, but possibilities for screening and selection of higher hydrogen producers might not have been fully exploited so far. Another option is to genetically modify *C. saccharolyticus* or other thermophiles in such a way that these micro-organisms will predominantly use the PP pathway for the fermentation of sugars.

Woodward et al. (2000) showed that a very high yield of 11.6 mol of hydrogen per mole of glucose 6-phosphate can be obtained in vitro on a small scale, which approached the theoretical maximum of 12 mol H_2 per mole glucose. The system consisted of the enzymes of the oxidative and regenerative branch of the pentose phosphate pathway, coupled to *P. furiosus* hydrogenase that uses $NADP^+$ as the electron carrier. The extremely high and unequalled hydrogen yield is very promising, especially for small production plants, although it remains to be seen whether the revenues of the very high yield will outweigh the costs for enzyme production and stabilization.

For a cost-effective industrial bioprocess, both hydrogen yield and hydrogen productivity will be of importance. With *C. saccharolyticus*, both the specific and the volumetric hydrogen production rate are highest at the higher growth rates in contrast to the hydrogen yield, which shows the opposite effect. Optimal hydrogen yields will decrease the contribution of the substrate costs to the total costs for biohydrogen production, whereas high productivities will have a positive effect on the costs for the installation by limiting its size. Ideally, a situation with low growth rates and high cell densities seems to be the most optimal. For larger scale biohydrogen processes, carbohydrate-rich biomass containing, for example, starch or lignocellulose is likely to be the substrate of choice. We already showed that biohydrogen production by extreme thermophilic bacteria is possible from sweet sorghum juice and hydrolysates of Miscanthus, paper sludge, potato peels and domestic organic waste (Claassen 2002). Further research is aimed at improving the biohydrogen production efficiency by using multiple biomass feedstocks as substrates, together with the development of bioreactors, which enable the optimal mix of high productivity and high yield (Claassen and de Vrije 2006).

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