Effect of carbon dioxide and temperature on H_2 producing cultures of the extreme thermophile, *Caldicellulosiruptor saccharolyticus*

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Abstract
Carbon dioxide could replace nitrogen gas as stripping gas to relieve cultures of the extreme thermophile, *Caldicellulosiruptor saccharolyticus*, of the inhibition by H_2, provided that the pH is kept below 7.0. Above this pH, ≥77 mM sodium bicarbonate will be introduced as an extra salt burden to the culture. High concentrations of solutes contribute to the inhibition of H_2 production. The underlying cause is most probably osmolality. As a consequence, using CO_2 as stripping gas might lower the maximum allowable acetate concentration in the culture, and therefore the productivity. With CO_2, lower yields of biomass and hydrogen were obtained, whereas the maximum specific growth rate was not affected. In a thermodynamic study an estimations of the activation enthalpies were made for both hydrogen production and thermal deactivation, being 45.9 kJ/mol and 160.1 kJ/mol, respectively. Hydrogen production showed a wider temperature range (estimated 40 - 90°C) than growth (49.6 – 85.7°C).

Keywords: extreme thermophile, *Caldicellulosiruptor saccharolyticus*, carbon dioxide, osmolality, activation enthalpy

1. Introduction
A hydrogen economy is getting ever more serious consideration and requires continuous hydrogen production preferably from renewable sources. Biomass is such a source since it is abundantly available. Several techniques are available for H_2 production from biomass, such as thermo-chemical and biological.
A cost effective biological hydrogen production plant is feasible, yet many improvements need to be achieved [1]. In a first fermentation step biomass, consisting mainly of sugars, is fermented by extreme thermophiles to H_2, CO_2 and small organic acids. An advantage of these organisms is that H_2 is obtained from sugars close to theoretical stoichiometry [2].
It is essential that the technology of separation and purification of the H_2 gas is cost-effective. Therefore, the application of an inert gas, such as N_2, as stripping gas should be avoided [3].
In this study the implications of using CO_2 as stripping gas were investigated in cultures of *Caldicellulosiruptor saccharolitycus*. Complications are expected, because of introduction of significant concentrations of bicarbonate. Besides undesired precipitations, higher salt concentrations might effect H_2 production and cell lysis [4].

Therefore, the effect of CO_2 on the performance of *C. saccharolyticus* was determined in batch cultures at the optimum pH (6.5) for growth. Sparging with CO_2 resulted in lower biomass and hydrogen production yields; however, more experiments are needed to find the reason behind this.
The temperature is one of the most important parameters in the thermofermentation, In our quest for the optimal process parameters, it was observed that *C. saccharolyticus* was quite sensitive to small changes in the temperature with respect to biomass yield and hydrogen production (Martens, van Niel and Claassen, unpublished results). Fluctuations of temperature in the bioreactor might happen and could therefore influence the performance of the fermentation. Therefore, the effect of the temperature on hydrogen production and growth of *C. saccharolyticus* was studied more closely. A thermodynamic study, including an Arrhenius plot, allowed estimation of activation enthalpies for hydrogen production and for thermal deactivation. Interestingly, hydrogen production showed a wider temperature range than growth.

2. Materials and methods

2.1 Organism and media

Caldicellulosiruptor saccharolyticus (DSM 8903) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Two media were used: a bicarbonate-buffered medium was applied in crimp seal flasks, because of its strong buffering capacity, and a slightly modified DSM640-medium (**http://www.dsmz.de/media/med640.htm)** was applied in the reactor to avoid undesired physicochemical reactions [4]. Sucrose (10 g/L) was used as the C- and energy source.

2.2 Growth and inhibition experiments

Cultures were grown in a jacketed 3 L-reactor (Applikon, Schiedam) at a working volume of 1 L. The pH was monitored by an Applikon Biocontroller 1030 and maintained at pH 6.5 (is neutral pH at 70ºC; the pH-electrode was calibrated at room temperature). The temperature was thermostatically kept at 70ºC and the stirring rate was 350 rpm. Prior to inoculation, the medium was sparged with 100% N_2 or 100% CO_2 at a flow rate of 7 L/h to remove the oxygen, and the medium was finally reduced by addition of dithiotreitol (DTT). The inoculum was grown overnight in the bicarbonate-buffered medium.
The effect of sucrose on exponential growing cells was studied in 120-mL crimp seal flasks. Cultures were grown in the reactor until exponential growth phase and aliquots of 20 mL culture were transferred directly into vacuum crimp seal flasks. During the transfer, the flasks were kept on ice to stop cell activity temporarily. Hydrogen production was regained within 10 min incubation in a water bath at 70ºC. Prior to incubation, sucrose was added in different concentrations to the flasks. The inhibition kinetics was determined by following hydrogen accumulation in the headspace for 2-3 h. The effect of sucrose on cell lysis was studied by following the OD of the culture for 2-3 h in a second series of 20 mL aliquots in 120-mL crimp seal flasks.

2.3 Analyses

Hydrogen was measured by GC (Packard 427, Chrompack, The Netherlands) using a RVS Mol Sieve 5A, 60/80 mesh, 3 m × 1/8" column. The temperature of the thermal conductivity detector, injector and column was 100°C, 80°C and 50°C, respectively.

N_2 was used as carrier gas. Sucrose and organic acids were analyzed by HPLC (Waters, The Netherlands) on a Shodex ionpak KC811 column (Waters, The Netherlands) at 80°C with differential refractometric detection and 3 mM H_2SO_4 as mobile phase (1 ml/min). The optical density was measured spectrophotometrically at 620 nm using a Pharmacia spectrophotometer. A relationship between OD and biomass dry weight (DW) was measured: 728 × OD_{620} mg/L.

2.4 Calculation and determination of dissolved CO_2 concentration
The unionized and ionized fractions of dissolved CO_2 were calculated at a temperature range of 65-75ºC. Model equations and parameter values of Stumm and Morgan [5] were applied. The amount of sodium hydroxide necessary for neutralizing bicarbonate at different pH was calculated and verified experimentally in sterile medium that was continuously sparged with 100% CO_2. The aliquote of 1 M sodium hydroxide solution required to set a certain pH was determined by weight using a balance.

2.5 Inhibition kinetics
For the determination of the kinetics of substrate inhibition a generally applicable equation of growth inhibition [6] was adapted to the noncompetitive inhibiting effect of the substrate on the hydrogen production rate r_H:

$$r_H = r_{H,MAX} \cdot (S/(K_S + S)) \cdot (1 - S/S_{crit})^n \quad (1)$$

with $r_{H,MAX}$ = maximum hydrogen production rate ($\mu l \cdot mg^{-1} \cdot h^{-1}$), K_S = the affinity coefficient for sucrose (2.07 mM), S = the concentration of sucrose (mM), S_{crit} = the critical sucrose concentration (mM) at which the inhibition is 100%, and n = the inhibition exponent.

2.6 Determination of the thermodynamic parameters
Cultures were grown in the reactor until exponential growth phase. Aliquots of 20 mL culture were transferred directly into two series of 120-mL crimp seal flasks. Hydrogen production was regained within 15 min of incubation in water baths at various temperatures. Growth and hydrogen production were followed for 5-6 hours. The rates of hydrogen production were used to calculate the values of the activation and deactivation enthalpy using the Arrhenius equation:

$$r_H = A \cdot \exp[-\Delta H_A/RT] \quad (2)$$

$$r_H = B \cdot \exp[-\Delta H^*/RT] \quad (3)$$

$$\Delta H_D = \Delta H_A + |\Delta H^*| \quad (4)$$

With r_H = hydrogen production rate, A and B = the Arrhenius pre-exponential factor, ΔH_A = the activation enthalpy [$kJ \cdot mol^{-1}$], ΔH_D = the deactivation enthalpy [$kJ \cdot mol^{-1}$], and R is the ideal gas constant [$J \cdot mol^{-1} \cdot K^{-1}$]. The thermodynamic approach [7] describes the hydrogen production rate as a function of the temperature:

$$r_{H,max} = A_0 \cdot \exp[-\Delta H_A^0/RT]/(1+C \cdot \exp[-\Delta H_D^0/RT]) \quad (5)$$

with A_0 as the Arrhenius pre-exponential factor linked to the activation entropy, C as the entropy contribution of the deactivation equilibrium, and ΔH_D^0 being the standard variation of deactivation enthalpy of an enzyme controlling a fundamental metabolic pathway.
The growth rate could be described as a function of temperature with the model proposed by Ratkowsky et al [8]:

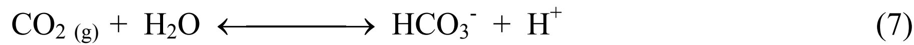
$$\mu_{max}(T) = \{b\cdot(T-T_{min})\cdot(1- \exp[c\cdot(T-T_{max})])\}^2 \qquad (6)$$

with the coefficients b and c having the units $K^{-1}h^{-0.5}$ and K^{-1}, respectively, and the minimum (T_{min}) and maximum (T_{max}) growth temperatures.

3. Results and discussion

3.1 Carbon dioxide solubility at elevated temperature

To use carbon dioxide as a stripping gas, complications in the form of ionized CO_2 may be introduced. At neutral pH, carbon dioxide is mainly present in two forms: CO_2/H_2CO_3 and HCO_3^-. These are in equilibrium according to the following reaction:



$$CO_{2\,(g)} + H_2O \longleftrightarrow HCO_3^- + H^+ \qquad (7)$$

The amount of bicarbonate can be calculated from the overall equilibrium constant (K_{ga} [M/atm]) of the reaction:

$$[HCO_3^-] = K_{ga} \cdot P_{CO2}/[H^+] \qquad (8)$$

with P_{CO2} being the partial pressure of carbon dioxide (atm). For this study, the value of K_{ga} should be known for a temperature of 70ºC. The overall equilibrium constant is dependent on the temperature according to:

$$K_{ga} = K_{ga}^S \cdot \exp\{-(\Delta H_r^0/R)\cdot(1/T - 1/T^S)\} \qquad (9)$$

with K_{ga}^S = overall equilibrium constant at standard temperature (T^S) (= $1.43 * 10^{-8}$ $M\cdot atm^{-1}$), ΔH_r^0 = standard enthalpy of the overall reaction (= -11.8 $kJ\cdot mol^{-1}$) and R = gas constant (= 8.3145 $J\cdot mol^{-1}\cdot K^{-1}$).
The nominal amount of $CO_{2\,(g)}$ is independent of the pH and hence remains constant, whereas bicarbonate is strongly dependent on the pH (Table 1). The concentrations of HCO_3^- and its counter ion, Na^+, increase with the pH. The amount of Na^+ measured from the titration with NaOH at 70°C was comparable to that predicted by the model (Table 1). As long as the pH is kept below 7.0, no substantial amounts of bicarbonate are introduced.

3.2 Carbon dioxide as stripping gas

The effects of CO_2 on growth and hydrogen production by sucrose fermenting cultures of *C. saccharolyticus* were compared to those in which N_2 was used as stripping gas (Table 2). The maximum specific growth rate was not affected by high partial pressures of CO_2, but the biomass yield and the stoichiometric ratio between hydrogen and sucrose decreased significantly. It needs yet to be elucidated why high concentrations of CO_2 affect these production yields.

3.3 Cause of inhibition by salts and sugars

Van Niel et al [4] described the substrate and product inhibition kinetics that are inherent to sugar fermentations with *C. saccharolyticus*. However, a cause of inhibition was not elucidated. In the present study a clue was found after determining the substrate inhibition kinetics in cultures exposed to high sucrose concentrations in their exponential phase (Fig. 1) instead of the lag phase. The critical concentrations of sucrose, sodium acetate, and sodium chloride, at which growth and hydrogen production is completely impaired, could now be compared (Table 3). The common cause of inhibition appears to be osmolality. However, this needs to be validated with more experiments, including statistical analysis. Due to this common cause, the critical concentration of sodium bicarbonate could be estimated (Table 3). With about 45 mM of monovalent ions (Na^+, K^+, NH_4^+) already present in the medium, the actual critical salt concentration will be approximately 410-420 mM. In former studies it was observed that high salt concentrations enhanced cell lysis [4]. In contrast, exposure of exponential growing cultures to high concentrations of sucrose for 2-3 h did not provoke enhanced cell lysis (results not shown).

3.4 Effect of temperature on growth and hydrogen production

The growth of *C. saccharolyticus* on sucrose and the accompanying hydrogen production were monitored at various incubation temperatures. The optimum temperature for both growth and hydrogen production was 71.8ºC. Values for the various thermodynamic parameters (Table 4) were determined applying equations (2) - (6) to the measured data (Fig. 2 and 3).The activation enthalpy of hydrogen production was lower than the value found for hydrogen production by *Enterobacter aerogenes* (67.3 $kJ \cdot mol^{-1}$; [9]), but was within the range obtained for alcohol fermentation by *Saccharomyces cerevisiae* (31.9 – 50.6 $kJ \cdot mol^{-1}$; [10]). The activation enthalpy of the thermal deactivation according to the Arrhenius model was similar to that for *E. aerogenes* [9] and suggests that an increased denaturation rate of a controlling enzyme is responsible for the decline in productivity. Compared to the Arrhenius model, the activation enthalpy found with the thermodynamical approach was similar, but the activation enthalpy for the thermal deactivation was significantly higher (Table 4). The latter result is similar to that obtained by Arni et al [10] with *S. cerevisiae*, and they concluded that the thermodynamic approach is less appropriate for whole cells, but that it works better for enzymes.

Hydrogen production, which is a catabolic activity, still continued beyond the minimum and maximum growth temperature (Fig. 3A). The latter suggests that the controlling enzyme takes part in an assimilating rather than a catabolic pathway. Equation (6) described the growth rate as function of temperature very well. The relationship between growth and hydrogen production can be expressed as a yield factor: $Y_{XH} = r_X/r_H$ (mg DW·mmol H_2^{-1}). The $Y_{XH}(T)$ as function of the temperature could be determined (Fig 3B) by dividing the data points of $\mu_{MAX}(T)$ by those of $r_H(T)$ at a temperature range of 50.3-78.3ºC shown in Fig. 3A:

$$Y_{XH}(T) = 0.46 * T - 23.27 \qquad R^2 = 0.917 \qquad (10)$$

The relationship given in equation (10) will be used in our future investigations on the effect of temperature fluctuations on *C. saccharolyticus* cultures with respect to growth and hydrogen production.

4. Conclusions

When using CO_2 as a gas for stripping H_2, the pH should be kept below pH 7.0 to prevent an additional salt burden in the form of sodium bicarbonate. High salt and sugar concentrations will each contribute to the osmolality of the medium. This will negatively affect growth and hydrogen production. A maximal critical solutes concentration of 410-420 mM was estimated for *C. saccharolyticus.* The results in this study show that sparging with CO_2 will lower the hydrogen production yield, but the reason remains unknown.

The optimal temperature for growth and hydrogen production were identical. This is not unusual since hydrogen is a primary product. The temperature range for the activity of hydrogen production was bigger than that for production of biomass. At the lower temperature scale this could be related to production of energy entirely for maintenance, whereas at the higher temperature scale this could indicate that the most temperature-sensitive enzyme takes part in an assimilating rather than a catabolic pathway.

5. Acknowledgements

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Table 1. Total dissolved amount of ionized and unionized CO_2 as a function of the pH in a system at 70ºC and 100% CO_2 atmosphere (pressure 10^5 Pa) and the concentration of Na^+ as counter ion according to the model and the measured titrated concentration.

pH	CO_2 (mM)	H_2CO_3 (mM)	HCO_3^- (mM)	CO_3^{2-} (mM)	Total C (mM)	Na^+ (mM)	Na^+ measured (mM)
6.28	12.21	0.03	14.6	8.83E-04	26.8	14.57	16.66
6.68	12.21	0.03	37.1	5.72E-03	49.3	37.08	32.66
6.98	12.21	0.03	73.9	2.27E-02	86.2	73.96	64.64
7.26	12.21	0.03	142	8.34E-02	154	141.70	123.83
7.69	12.21	0.03	375	5.85E-01	388	376.19	276.46

Table 2. Comparison of N_2 and CO_2 as stripping gases in batch cultures of *C. saccharolyticus* growing on sucrose at pH 6.5.

Stripping gas	μ_{MAX} (h^{-1})	H_2/sucrose	Yield (g DW/mol sucrose)
N_2	0.081 ± 0.003	6.6	45.1
CO_2	0.077 ± 0.006	3.9	34.2

Table 3. Determined critical concentrations of sucrose, sodium acetate and sodium chloride and estimated critical concentration of sodium bicarbonate and their corresponding osmolality.

Inhibitor	Critical concentration (mM)	Osmolality ($mOs \cdot kg^{-1}$)*)
Sucrose	474 ± 32	589 ± 44
Sodium acetate	365 ± 30	737 ± 63
Sodium chloride	344 ± 35	623 ± 66
Sodium bicarbonate*)	372	622

*) Handbook of Physics and Chemistry (1975).

Table 4. Determination of the values of the thermodynamic parameters of hydrogen production and growth of *C. saccharolyticus*.

Parameter		Value	Equation
ΔH_A	$kJ \cdot mol^{-1}$	45.9	(2)
ΔH_D	$kJ \cdot mol^{-1}$	160.1	(3)-(4)
ΔH_A^0	$kJ \cdot mol^{-1}$	46.5	(5)
ΔH_D^0	$kJ \cdot mol^{-1}$	194.3	(5)
T_{min}	°C	49.6	(6)
T_{max}	°C	85.7	(6)

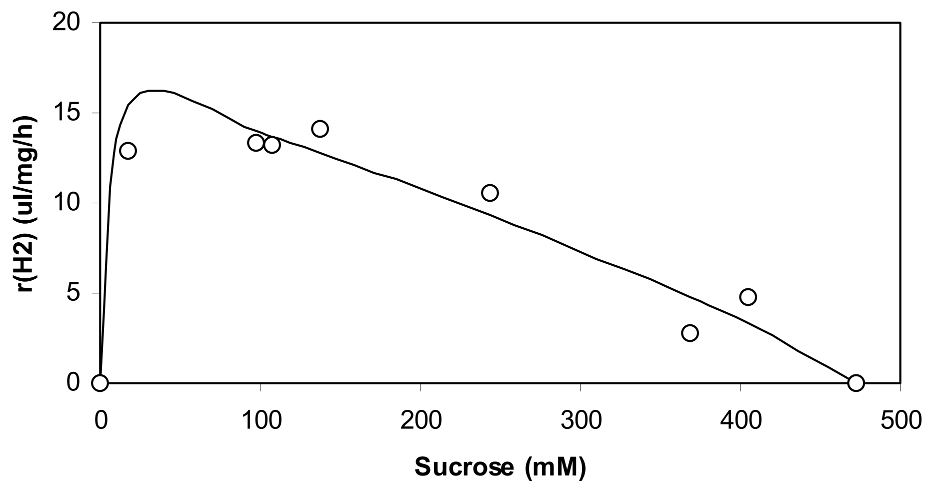

Figure 1. Inhibition by sucrose of hydrogen production by exponential growing cells of *C. saccharolyticus*. The line is fitted through the data points using equation (1).

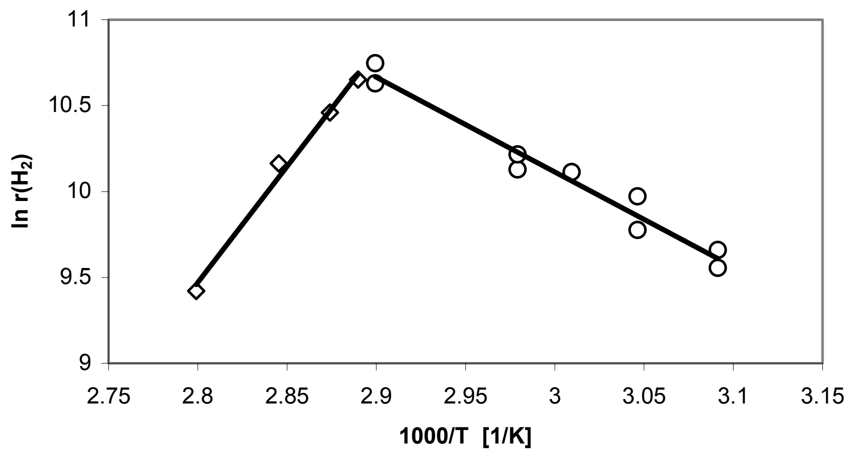

Figure 2. Arrhenius plot for the evaluation of activation enthalpies of both hydrogen production by *C. saccharolyticus* (equation 2) and thermal deactivation (equation 3).

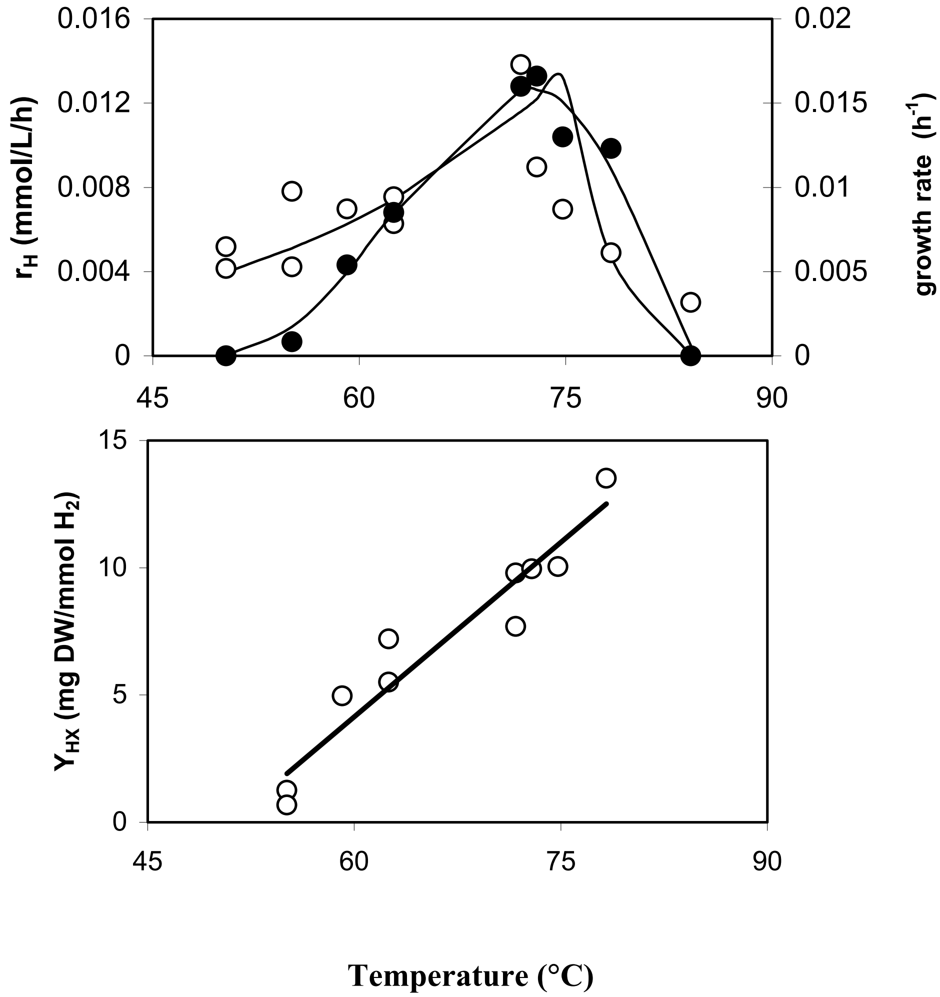

Figure 3. A. Estimation of the thermodynamic parameters of both fermentation and thermal deactivation according to the thermodynamic approach (equation 5; with $A_0 = 1.27 \cdot 10^5$ and $C = 9.5 \cdot 10^{239}$) (o). Estimation of the minimum and maximum growth temperatures (equation 6; with b=0.802 and c= 0.058) (•). B. Yield of biomass per hydrogen produced as function of the temperature. Line is best fit through the data points.