



# LUND UNIVERSITY

## Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*.

van Niel, Ed; Claassen, P A M; Stams, A J M

*Published in:*  
Biotechnology and Bioengineering

*DOI:*  
[10.1002/bit.10463](https://doi.org/10.1002/bit.10463)

2003

[Link to publication](#)

*Citation for published version (APA):*  
van Niel, E., Claassen, P. A. M., & Stams, A. J. M. (2003). Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. *Biotechnology and Bioengineering*, 81(3), 255-262. <https://doi.org/10.1002/bit.10463>

*Total number of authors:*  
3

### General rights

Unless other specific re-use rights are stated the following general rights apply:  
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00



# Substrate and Product Inhibition of Hydrogen Production by the Extreme Thermophile, *Caldicellulosiruptor saccharolyticus*

Ed W. J. van Niel,<sup>1</sup> Pieter A. M. Claassen,<sup>2</sup> Alfons J. M. Stams<sup>1</sup>

<sup>1</sup>Laboratory for Microbiology, Wageningen University and Research Center, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands; telephone: +31 317 483101; fax: +31 317 483829; e-mail: ed.vanniel@algemeen.micr.wau.nl

<sup>2</sup>Wageningen University and Research Center, Agrotechnological Research Institute (ATO B.V.), Department of Bioconversion, P.O. Box 17, 6700 AA Wageningen, The Netherlands

Received 23 October 2001; accepted 19 June 2002

DOI: 10.1002/bit.10463

**Abstract:** Substrate and product inhibition of hydrogen production during sucrose fermentation by the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus* was studied. The inhibition kinetics were analyzed with a noncompetitive, nonlinear inhibition model. Hydrogen was the most severe inhibitor when allowed to accumulate in the culture. Concentrations of 5–10 mM H<sub>2</sub> in the gas phase ( $\equiv$  partial hydrogen pressure (pH<sub>2</sub>) of  $(1-2) \cdot 10^4$  Pa) initiated a metabolic shift to lactate formation. The extent of inhibition by hydrogen was dependent on the density of the culture. The highest tolerance for hydrogen was found at low volumetric hydrogen production rates, as occurred in cultures with low cell densities. Under those conditions the critical hydrogen concentration in the gas phase was 27.7 mM H<sub>2</sub> ( $\equiv$  pH<sub>2</sub> of  $5.6 \cdot 10^4$  Pa); above this value hydrogen production ceased completely. With an efficient removal of hydrogen sucrose fermentation was mainly inhibited by sodium acetate. The critical concentrations of sucrose and acetate, at which growth and hydrogen production was completely inhibited (at neutral pH and 70°C), were 292 and 365 mM, respectively. Inorganic salts, such as sodium chloride, mimicked the effect of sodium acetate, implying that ionic strength was responsible for inhibition. Undissociated acetate did not contribute to inhibition of cultures at neutral or slightly acidic pH. Exposure of exponentially growing cultures to concentrations of sodium acetate or sodium chloride higher than ca. 175 mM caused cell lysis, probably due to activation of autolysins. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* 81: 255–262, 2003.

**Keywords:** hydrogen; product inhibition; substrate inhibition; kinetics; cell lysis

## INTRODUCTION

Research on alternative energy sources has gained renewed interest, due to global awareness of accumulated CO<sub>2</sub> in the atmosphere as a potential cause of climate change (Benemann, 1996). Biomass, which is widely available, is a renewable resource for alternative fuel production (Claassen et al., 1999). An interesting option is the conversion of biomass to hydrogen, a carbon-free energy carrier. There are two technical processes to generate hydrogen from biomass, i.e., gasification and fermentation. Both processes have their own merits, but when “pure” hydrogen is desired, e.g., for fuel cells, microbial fermentation is especially attractive.

Hydrogen is produced by both mesophiles and (hyper) thermophiles, the latter showing superior production rates (Adams, 1990). In general, less variety in fermentation by-products is seen under thermophilic conditions (Schönheit and Schäfer, 1995). These properties make application of (hyper)thermophiles for hydrogen production economically and technically interesting. However, little research has been done on fermentations by these thermophiles with respect to the formation of hydrogen.

Here we present the effects of inhibitors and the kinetics of inhibition on hydrogen production by the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus* during growth on sucrose. This organism was selected for its ability to use many (poly)saccharides, including cellulose, for growth (Donnison et al., 1989; Rainey et al., 1994). It has been shown previously that it converts sugars to hydrogen, acetate, lactate, and traces of ethanol (Rainey et al., 1994).

The aim of this study was to investigate how products affect hydrogen production from sugar fermentation by *C. saccharolyticus*. Inhibition of growth by hydrogen was observed in a variety of fermentative microorganisms, includ-

Correspondence to: Ed W. J. van Niel  
Contract grant sponsor: European Commission  
Contract grant number: QLRT-1999-01267

ing (hyper)thermophiles (Wiegel and Ljungdahl, 1981; Malik et al., 1989; Adams, 1990; Schröder et al., 1994). A part of the inhibition mechanism was identified as product inhibition of the H<sub>2</sub>-evolving hydrogenase (Adams et al., 1981; Gottschalk, 1986). So far, inhibition of extreme or (hyper)thermophiles by acetate and other organic compounds was only studied for *Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl, 1981), but it remained unknown how it was kinetically related to growth or hydrogen production. Like other organic acids, acetate is known to impair growth of many microorganisms (Lasko et al., 2000). It has been described that in both the undissociated or dissociated form of these compounds may act as uncouplers of growth (Wang and Wang, 1984; Booth, 1985).

## MATERIALS AND METHODS

### Organism and Media

*C. 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. It consisted of (per L) KH<sub>2</sub>PO<sub>4</sub> 0.41 g, Na<sub>2</sub>HPO<sub>4</sub> 0.53 g, CaCl<sub>2</sub> 0.11 g, MgCl<sub>2</sub> 0.1 g, NH<sub>4</sub>HCO<sub>3</sub> 0.44 g, NaHCO<sub>3</sub> 3.73 g, Na<sub>2</sub>S · 9H<sub>2</sub>O 0.5 g, yeast extract 1 g, resazurin 1 mg, vitamin solution 1 mL, and trace element solution 1 mL. Unless stated otherwise, sucrose was used in a concentration of 10 g/L. The gas phase contained 80% N<sub>2</sub> and 20% CO<sub>2</sub>, and the pH was 7.2. A slightly modified DSM640 medium (<http://www.dsmz.de/media/med640.htm>) was used in the reactor to avoid undesired chemical reactions or precipitations that were observed with the bicarbonate-buffered medium under a 100% N<sub>2</sub> gas phase. The changes consisted of the omission of Trypticase and replacement of cysteine-HCl (0.6–0.75 g/L) by dithiothreitol (DTT) (0.2 g/L). The modified DSM640 medium at a pH 7 consisted of (per L) K<sub>2</sub>HPO<sub>4</sub> 1.5 g, KH<sub>2</sub>PO<sub>4</sub> 0.75 g, NH<sub>4</sub>Cl 0.9 g, NaCl 0.9 g, MgCl<sub>2</sub> · 6H<sub>2</sub>O 0.4 g, DTT 0.2 g, yeast extract 1 g, resazurin 1 mg, vitamins solution 1 mL, and trace element solution 1 mL. Sucrose (10 g/L) was used as the C and energy source. The vitamin solution consisted of (mg/L) biotin 20, folic acid 20, pyridoxine-HCl 100, riboflavin 50, thiamin-HCl 50, nicotinamide 50, cobalamin 50, *p*-aminobenzoic acid 50, lipoic acid 50, and pantothenic acid 50. The trace elements solution SL-10 consisted of (per L) FeCl<sub>2</sub> · 4H<sub>2</sub>O 1.5 g, ZnCl<sub>2</sub> 70 mg, MnCl<sub>2</sub> · 4H<sub>2</sub>O 0.1 g, H<sub>3</sub>BO<sub>3</sub> 6 mg, CoCl<sub>2</sub> · 6H<sub>2</sub>O 0.19 g, CuCl<sub>2</sub> · 2H<sub>2</sub>O 2 mg, NiCl<sub>2</sub> · 6H<sub>2</sub>O 24 mg, Na<sub>2</sub>MoO<sub>4</sub> · H<sub>2</sub>O 36 mg, Na<sub>2</sub>WO<sub>4</sub> 15 mg, Na<sub>2</sub>SeO<sub>3</sub> · 5H<sub>2</sub>O 15 mg.

### Batch Growth and Inhibition by Hydrogen

Cultures were grown in a jacketed 3L-reactor (Applikon, Schiedam, The Netherlands) at a working volume of 1 L.

The pH was monitored by an Applikon Biocontroller 1030 and maintained at pH 6.5 (pH is neutral at 70°C; the pH electrode was calibrated at room temperature). The temperature was thermostatically kept at 70°C, and the stirring rate was set to 350 rpm. Prior to inoculation, the medium was sparged with N<sub>2</sub> at a flow rate of 7 L/h to remove the oxygen, and the medium was finally reduced by addition of DTT. The inoculum was grown overnight in the carbonate-buffered medium. Rapid growth was obtained with continuous flushing with N<sub>2</sub> to remove the hydrogen. Terminating the flow of N<sub>2</sub> and closing the gas effluent enabled accumulation of H<sub>2</sub> in the gas phase.

### Inhibition by Substrate and Salts

The effect of sucrose and several salts was studied in 45-mL cultures that were incubated overnight in 250-mL crimp seal flasks. The following compounds were added at early exponential growth phase: sucrose (0–100 g/L = 0–292 mM); sodium acetate (0–300 mM); potassium acetate (0–240 mM); sodium lactate (0–240 mM); sodium chloride (0–300 mM); potassium chloride (0–240 mM). At early exponential phase the pH was about 6.2–6.5 (at room temperature) and was hardly influenced by the added compounds (300 mM sodium acetate affected the pH by only 0.01 unit). To determine the inhibition kinetics of sodium acetate and sodium chloride, cultures were grown in the reactor until the exponential growth phase. Aliquots of 20 mL were then transferred directly into vacuum 120-mL crimp seal flasks. During the transfer, the crimp seal flasks were held on ice to stop cell activity temporarily. Transfer into vacuum flasks had no damaging effect on the cells, as was measured previously. Hydrogen production was regained within 10 min of incubation in a waterbath at 70°C. Prior to incubation, sodium acetate or sodium chloride was added in different concentrations to the flasks. The pH (measured at room temperature) at the start of the experiments was ca. 7.2 and dropped to minimally 6.1 in cultures that kept the highest H<sub>2</sub> production rate. The inhibition kinetics were determined by following hydrogen accumulation in the headspace for 2–3 h. The effect of pH on inhibition by sodium acetate was studied in two series with different initial pH. In one series the headspace of exponentially growing cultures was replaced by N<sub>2</sub>/CO<sub>2</sub> (80%/20% v/v), keeping the initial pH at 6.5. In the second series the headspace was replaced by N<sub>2</sub>, changing the initial pH to 7.2. Subsequently, different aliquots of a 3-M sodium acetate solution were added to each series, and hydrogen production was followed for 2 h.

### Cell Lysis Kinetics and Mechanism

Exponentially growing cells were drawn from the reactor (kept at pH 7.2) using vacuum 120-mL crimp seal flasks, which were kept on ice to stop activity temporarily. A range of concentrations of sodium acetate (0–300 mM) or sodium chloride (0–300 mM) were added to the flasks, which were subsequently incubated at 70°C. The optical density and

hydrogen production were followed for 2.5 h in separate series. To clarify the mechanism of cell lysis, cultures were incubated overnight in crimp seal flasks at 70°C. To inhibit protein synthesis (including autolysins), chloramphenicol (50 µg/mL) was added to cultures in early and in late exponential growth stages 30 min prior to addition of 300 mM sodium acetate. The controls did not contain chloramphenicol. Cell lysis was measured by following the OD of the culture.

## Analyses

The pH was measured at room temperature in all cases; a pH of 7.0 at room temperature had a value of 6.5 at 70°C. Hydrogen was measured on a 406 Packard gas chromatograph equipped with a thermal conductivity detector (TCD, 100 mA). The gases were separated at 100°C on a molecular sieve column (13×, 180 cm by ¼ inch, 60–80 mesh) with argon as the carrier gas. OD was measured spectrophotometrically at 620 nm using a Hitachi U-1100 spectrophotometer. Cells were counted with a phase contrast microscope using a Bürker-Türk counting chamber. A relation between OD and cell number was calculated: [biomass] = (568.75 · [OD]<sub>620</sub>) mg/L ( $R^2 = 0.995$ ). Sucrose was measured with the anthron/sulfuric acid method (Trevelyan and Harrison, 1952). Protein was determined according to Bradford (1976). Organic acids were analyzed by HPLC (ThermoQuest, USA) on a column for organic acids (Polyspher OA HY, Merck, Germany), and detected by differential refractometry. The mobile phase was 0.01 N H<sub>2</sub>SO<sub>4</sub> and had a flow rate of 0.6 mL/min. The working temperature was 60°C.

## Determination of Kinetic Parameters

A generally applicable equation of growth inhibition (Han and Levenspiel, 1988) was adapted to the noncompetitive inhibiting effect of the compounds on the hydrogen production rate:

$$r(\text{H}_2) = r(\text{H}_2)_{\text{MAX}} * (1 - C/C_{\text{CRIT}})^n * S/(S + K_S) \quad (1)$$

with  $r(\text{H}_2)$  being the production rate of H<sub>2</sub> (mM H<sub>2</sub>/h);  $r(\text{H}_2)_{\text{MAX}}$  the maximum production rate of H<sub>2</sub> (mM H<sub>2</sub>/h);  $C$  the concentration of the inhibiting compound (mM);  $C_{\text{CRIT}}$  the critical concentration of the inhibiting compound at which H<sub>2</sub> production ceases (mM);  $K_S$  the apparent half-velocity constant for the substrate (mM);  $S$  is the substrate concentration (mM); and  $n$  is the degree of inhibition. For cases where the substrate concentration was not limiting ( $S \gg K_S$ ), Eq. (1) could be simplified to:

$$r(\text{H}_2) = r(\text{H}_2)_{\text{MAX}} * (1 - C/C_{\text{CRIT}})^n \quad (2)$$

Values of the parameters  $n$  and  $C_{\text{CRIT}}$  were found by data-fitting using the nonlinear least-squares regression method (NLSR-method) (with Excel, Microsoft). The standard error

in each parameter value was calculated using the Gaussian method as described by Robinson (1985).

Batch growth could be described according to the exponential growth equation:

$$X = X_0 * \exp^{\mu * (t - t_0)} \quad (3)$$

where  $X$  = biomass (g DW/L) and  $X_0$  is biomass at time  $t_0 = 0$ ; time =  $t$  (h); and  $\mu$  is the growth rate (h<sup>-1</sup>) according to Monod multiplied by several inhibition terms:

$$\mu = \mu_{\text{MAX}} * (S/(K_S + S)) * \{(1 - C_1/C_{1,\text{CRIT}})^n * (1 - C_2/C_{2,\text{CRIT}})^m\} \quad (4)$$

Cell lysis kinetics could be described by a modification of the equation of Han and Levenspiel (1988), whereby it is assumed that the compound stimulates cell lysis:

$$k = k_{\text{MAX}} * (C/C_{\text{CRIT}})^p \quad (5)$$

where  $k$  is the specific rate of lysis (h<sup>-1</sup>),  $C$  the stimulator concentration (mM) according to a stimulation term, with  $C_{\text{CRIT}}$  being the concentration at which stimulation is maximal,  $k_{\text{MAX}}$  (h<sup>-1</sup>). Exponent  $p$  is the degree of lysis stimulation. Cell lysis in the presence of growth can hence be described as a combination of Eqs. (4) and (5):

$$\mu = \mu_{\text{MAX}} * (S/(K_S + S)) * (1 - C_1/C_{1,\text{CRIT}})^n * (1 - C_2/C_{2,\text{CRIT}})^m - k_{\text{MAX}} * (C/C_{2,\text{CRIT}})^p \quad (6)$$

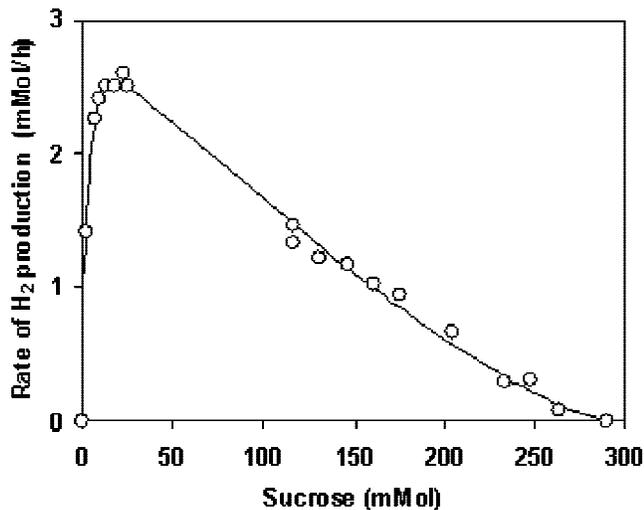
## RESULTS

### Inhibition by Sucrose

The effect of the concentration of sucrose on the hydrogen production rate was analyzed by determining the growth rates in cultures growing on 1.8–300 mM sucrose. All results (19 data points) were used to calculate the half-velocity constant for sucrose ( $K_S = 2.09 \pm 0.07$  mM), and the degree of inhibition ( $n = 1.39 \pm 0.04$ ) ( $R^2 = 0.96$ ). The critical sucrose concentration ( $[S]_{\text{crit}} = 292$  mM) was hereby estimated graphically from the data points shown in Fig. 1. After differentiation of Eq. (2) the concentration of sucrose yielding the maximum apparent growth rate was 19 mM. In all other experiments 29 mM sucrose was used, allowing thus for 98% of the maximum obtainable growth rate.

### Inhibition by Hydrogen

The effect of the concentration of hydrogen on growth and hydrogen production was studied in the chemostat. Hydrogen was allowed to accumulate in three separate cultures each at a different growth stage: lag phase (at the time of inoculation), early exponential phase, and mid-exponential phase. In all experiments the stirrer speed was kept at 350 rpm. In all three cases the concentration of H<sub>2</sub> in the gas phase increased with time according to a polynomial of the second order (Fig. 2A). The growth rate became linear and



**Figure 1.** Effect of the sucrose concentration on the hydrogen production rate of *C. saccharolyticus* during exponential growth. The line represents the best fit of Eq. (1) through the data points.

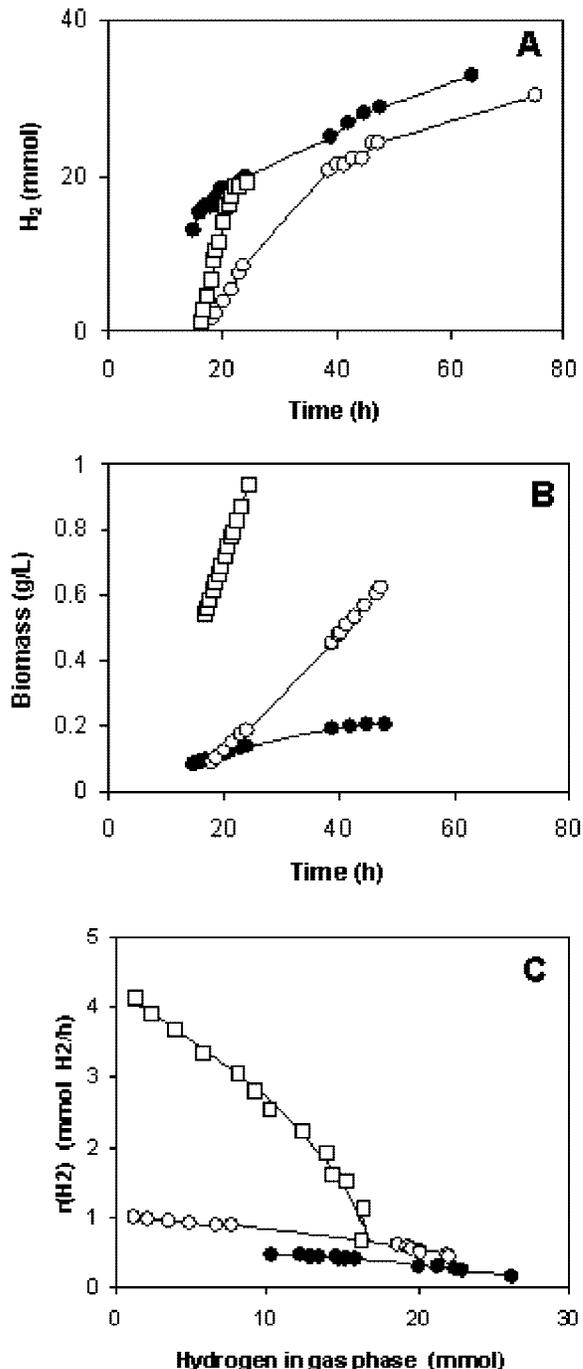
remained constant (Fig. 2B) despite the diminishing rate of  $H_2$  production. In the case where  $H_2$  accumulated during the lag phase growth stopped eventually (Fig. 2B) and sucrose consumption ceased (result not shown).

The  $H_2$  production rate can be calculated from the data in Fig. 2A. A plot of  $H_2$  production against the hydrogen concentration revealed that maximum tolerance to  $H_2$  was different for each growth phase (Fig. 2C). With accumulation of  $H_2$  in the lag phase and early and mid-exponential phases, the critical  $pH_2$  decreased from  $27.74 \pm 0.43$  mM ( $R^2 = 0.98$ ) to  $25.12 \pm 0.82$  mM ( $R^2 = 0.95$ ), and  $17.28 \pm 0.35$  mM ( $R^2 = 0.79$ ) (calculated from the data points in Fig. 2C with the NLSR method), respectively. Thus, inhibition by  $H_2$  became more pronounced when growth progressed. This was not a result of sucrose limitation, because the residual concentration was between 7 and 29 mM. Moreover, in this concentration range the growth rate varied only 10% due to substrate inhibition (Fig. 1).

An example of a time course of fermentation is given in Fig. 3 for a culture with accumulation of  $H_2$  in early exponential phase. Both acetate and  $H_2$  increased continuously during the entire experiment. Lactate production started at a hydrogen concentration in the gas phase of 5–10 mM ( $\equiv pH_2$  of  $(1-2) \cdot 10^4$  Pa) and became the dominant fermentation product. Ethanol was produced only in trace amounts (Fig. 3). The fermentation patterns in the other cases were similar, including the onset of lactate production at 5–10 mM  $H_2$ .

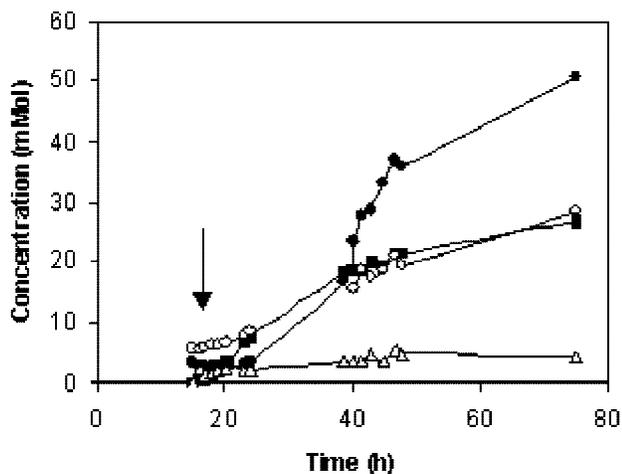
### Inhibition by Sodium Acetate and Other Salts

Aliquots of exponentially growing cells in the reactor were transferred directly into vacuum crimp seal flasks. Prior to incubation at 70°C, different acetate concentrations were added to the flasks. The hydrogen production rates in the



**Figure 2.** (A) Time course of hydrogen accumulation in the gas phase during three stages of growth in batch cultures of *C. saccharolyticus*. (B) Effect of hydrogen accumulation on growth. (C) Effect of hydrogen concentration on hydrogen production rate of the culture. The lines represent the best fits of Eq. (2) through the data points. Hydrogen accumulation was allowed to commence during: lag phase (●), early exponential phase (○), and mid-exponential phase (□).

flasks were determined in the first few hours of incubation and plotted against the acetate concentration (Fig. 4A). Estimating parameter values for the critical acetate concentration and  $n$  led to unrealistic values for both. Therefore, the degree of inhibition ( $n$ ) was set to 1, hence a critical acetate concentration of  $365 \pm 30$  mM ( $R^2 = 1$ ) was found.



**Figure 3.** Production of acetate (○), H<sub>2</sub> (in the gas phase) (■), lactate (●), and ethanol (△) during hydrogen accumulation in the gas phase of a batch of *C. saccharolyticus* on sucrose at the onset of the exponential phase. Arrow indicates start of hydrogen accumulation.

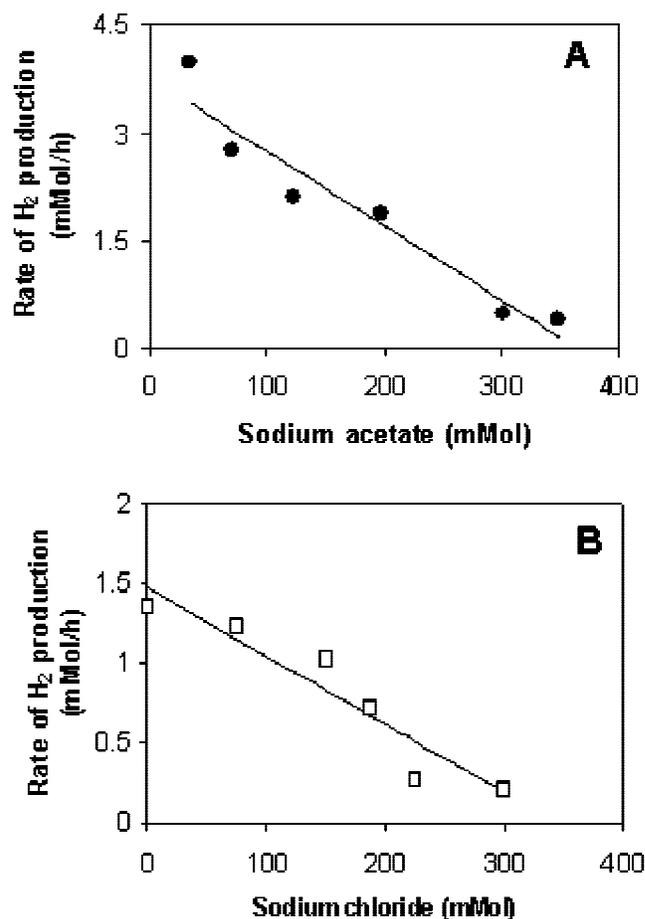
Subsequently, the nature of inhibition was elucidated. Firstly, the inhibition kinetics with sodium chloride were determined, and were found to be identical to that of acetate; with  $n$  set to 1, estimation of critical NaCl concentration becomes  $344 \pm 35$  mM ( $R^2 = 1$ ) (Fig. 4B). Secondly, the contribution of undissociated acetate was studied at two pH values (7.2 and 6.5), but no significant difference in critical acetate concentration was found (results not shown). It can thus be concluded that the increase in ionic strength was completely responsible for inhibition at the applied pH.

The inhibitory effect by high ionic strength was further demonstrated with other organic and inorganic salts. Sodium and potassium were compared as counter ions to acetate and chloride. There was only a difference with respect to the values of  $n$  (Table I). The critical concentrations for sodium acetate and sodium chloride found here were lower than in Fig. 4A and B, possibly due to the use of a sodium bicarbonate buffer (44 mM). The values in Table I were not corrected for this sodium source, as the aim here was to compare the effect of the added salts only.

### Cell Lysis

The effect of salt concentration on growth was checked by measuring the final OD. It appeared that with concentrations of inhibitor up to about 200 mM growth still took place, but beyond this concentration cell densities had decreased suggesting cell lysis (results not shown). After 2.5 h of incubation with inhibitor, cell lysis was maximally 30%. Cell lysis was further indicated by the presence of protein in the culture fluid. Cell lysis could be prevented by addition of chloramphenicol 1 h prior to addition of high salt concentrations (results not shown).

The kinetics of cell lysis was studied at different sodium acetate concentrations. Exponentially growing cells were sampled from the reactor as before and exposed to different



**Figure 4.** Effect of the concentration of sodium acetate (A) and sodium chloride (B) on the production rate of hydrogen (as measured in the gas phase) by *C. saccharolyticus* on sucrose. The lines represent best fits of Eq. (2) through the data points, with the value for  $n$  set to 1.

concentrations of sodium acetate. The optical density was followed in the first 2.5 h of incubation (Fig. 5A). The increase of OD was maximal in the absence of acetate, and declined with increasing sodium acetate concentration. Above 200 mM acetate, the OD declined from the start (Fig. 5A). However, even when cell lysis dominated, H<sub>2</sub> was still produced, as was measured in a separate series (Fig. 5B). Plotting the rate of increase of OD against sodium acetate concentration revealed a net cell lysis, assuming that growth was arrested completely, beyond 175 mM (Fig. 5C). Eq. (6) was fitted through the five data points, whereby values found in previous experiments were taken for  $C_{\text{CRIT1}}$  and  $n$ : 365 mM and 1, respectively. In several experiments it was seen that the value of  $C_{\text{CRIT2}}$  was close to that of  $C_{\text{CRIT1}}$  (results not shown), and hence was assumed to be identical. With the NLSR method the following parameter values were calculated:  $\mu_{\text{MAX}} = 0.13 \pm 0.01$  h<sup>-1</sup>;  $k_{\text{MAX}} = 0.25 \pm 0.06$  h<sup>-1</sup>;  $p = 2.8 \pm 0.6$  ( $R^2 = 0.94$ ).

### Growth in Batch Culture

It is clear from this work that several compounds present in the culture broth may, each to its own extent, negatively

**Table I.** Estimation of parameter values with standard errors and correlation coefficients of several salts inhibiting hydrogen production by growing cultures of *C. saccharolyticus* on sucrose in crimp seal flasks, plus the number of data points used for each estimation.

Inhibitor	Critical inhibitor concentration (mM)	<i>n</i>	<i>R</i> <sup>2</sup>	No. of datapoints
Sodium acetate	192 ± 23	1.42 ± 0.31	0.99	8
Potassium acetate	206 ± 12	0.58 ± 0.15	0.81	12
Sodium lactate	184 ± 7	0.45 ± 0.09	0.90	5
Sodium chloride	216 ± 10	0.56 ± 0.14	0.98	6
Potassium chloride	250 ± 11	0.50 ± 0.11	0.98	8

influence growth and metabolism of *C. saccharolyticus*. The hydrogen concentration is by far the most sensitive parameter, but when it is continuously removed from the culture other compounds may limit hydrogen production. To determine this, growth was followed in a culture that was continuously sparged with N<sub>2</sub>. It appeared that cells entered the stationary phase before all sucrose became limiting (Fig. 6). The p<sub>H<sub>2</sub></sub> remained below 2%, and acetate and H<sub>2</sub> were the main products of fermentation. Protein accumulated in the culture fluid up to 30 mg/L from the early exponential growth phase onward. This corresponded to about 60 mg biomass/L, meaning that ca. 7% of the total biomass produced was lost by cell lysis. From the increase in biomass, an apparent maximum specific growth rate of 0.073 h<sup>-1</sup> was calculated with Eq. (3). The best equation plotted through the growth data points of the exponential phase was obtained when inhibition by both sucrose and acetate were taken into account [Eq. (4)]. For the calculations initial values were chosen for the critical sucrose concentration, degree of inhibition, and *K<sub>S</sub>* for sucrose (292 mM, 1.39, and 2.09 mM, respectively, from Fig. 1), the critical acetate concentration and *n* (365 mM and 1, respectively, from data in Fig. 4A). Hence a value of 0.13 ± 0.04 h<sup>-1</sup> (*R*<sup>2</sup> = 0.96) was found for the true maximum growth rate, nearly twice the apparent maximum growth rate. No realistic parameter values were obtained trying to fit an alternative model, containing cell lysis kinetics [Eq. (6)]. This suggested that cell lysis was not the cause of growth inhibition but is rather a consequence of it under the conditions tested.

## DISCUSSION

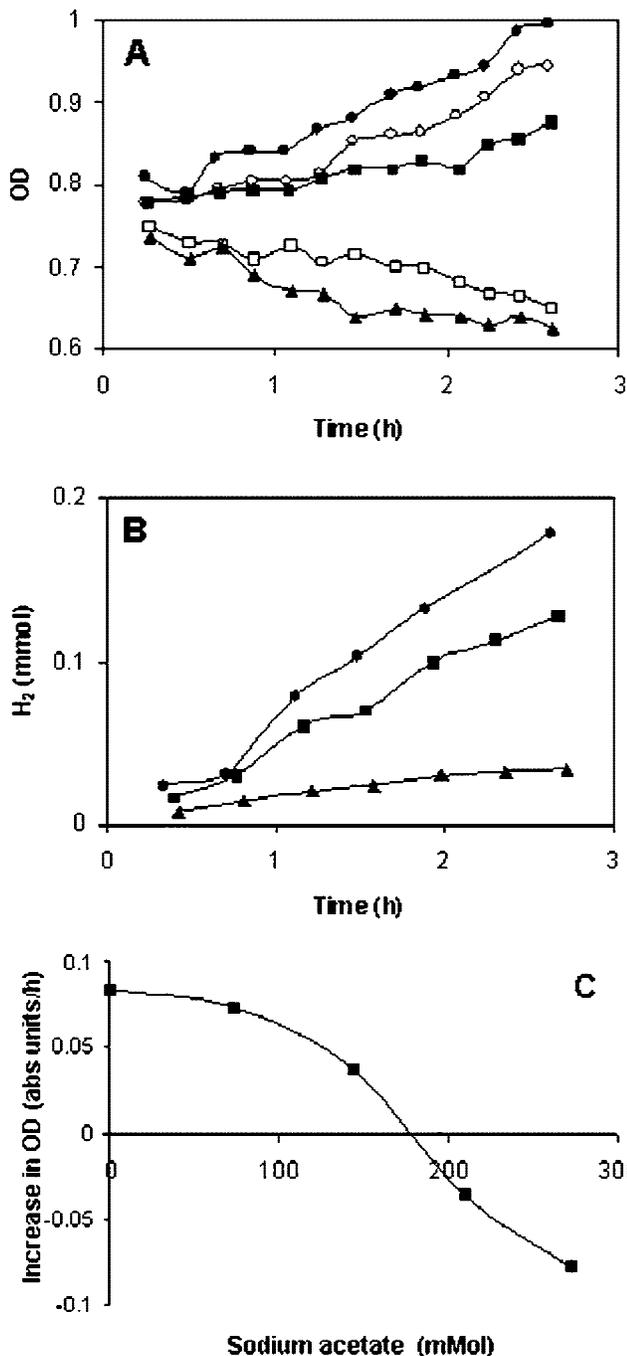
Hydrogen is known to restrict growth of thermophiles, and it inhibits its own production at the level of hydrogenase (Adams, 1990). As a consequence, reducing equivalents accumulate in the cell, and thus diminish the metabolic rate. Thermophiles usually possess some escape routes to dispose of reductants to prevent obstructions in their metabolic flux. One such route is production of more reduced organic compounds like lactate, ethanol or alanine (Janssen and Morgan, 1992; Kengen and Stams, 1994; Kengen et al., 1996). An alternative route is the reduction of S<sup>0</sup> to sulfide, provided sulfur is present in the culture medium (Malik et al., 1989).

Growth inhibition of thermophiles by hydrogen has been described previously (Fiala and Stetter, 1986; Huber et al.,

1986; Malik et al., 1989) but was never studied in detail. Growth, to which H<sub>2</sub> production is directly coupled, of *Thermotoga maritima* on glucose (Schröder et al., 1994) and *Pyrococcus furiosus* on pyruvate (Schäfer and Schönheit, 1991) was constrained significantly at partial hydrogen pressures (p<sub>H<sub>2</sub></sub>) of 2 · 10<sup>3</sup> Pa and 1.6 · 10<sup>3</sup> Pa, respectively. At these p<sub>H<sub>2</sub></sub>, the rate of hydrogen production of cultures of *C. saccharolyticus* were inhibited by only 7%. Inhibition of H<sub>2</sub> formation by this organism became significant at p<sub>H<sub>2</sub></sub> ≥ 10<sup>4</sup> Pa, at which level lactate production initiated (Fig. 2C). Hydrogen and acetate continued to be produced up until the critical p<sub>H<sub>2</sub></sub>, indicating that the metabolic shift to lactate was not complete.

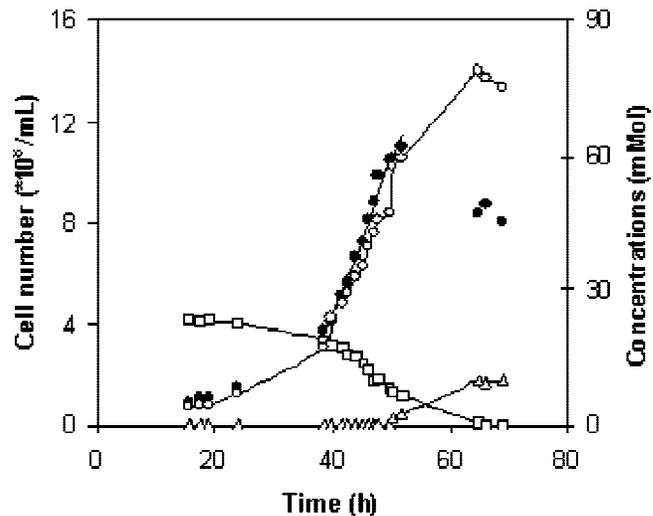
Growth of *C. saccharolyticus* and H<sub>2</sub> production ceased completely at a p<sub>H<sub>2</sub></sub> of 5.7 · 10<sup>4</sup> Pa (Fig. 2B,C), a value close to that found for *Thermoanaerobacter ethanolicus* (7.5 · 10<sup>4</sup> Pa; Wiegel and Ljungdahl, 1981). This critical value for *C. saccharolyticus* was found at cell densities in the order of 10<sup>8</sup> cells/mL. However, at cell densities 10 times higher and similar operational conditions in the reactor, the critical p<sub>H<sub>2</sub></sub> had declined by about 40% (Fig. 2C). This might be due to a slight hold-up of H<sub>2</sub> in the liquid caused by a limitation in the mass transfer to the gas phase (Paus et al., 1990). The hold-up increases with cell density, hence creating an inhibitory effect at a lower apparent p<sub>H<sub>2</sub></sub>. An indication of the critical dissolved hydrogen concentration can be calculated from data of hydrogen accumulation during the lag phase. The hydrogen production rate (calculated by multiplying the acetate production rate by a stoichiometric factor of 2) was in this case equal to the mass transfer rate for hydrogen (calculated from H<sub>2</sub> accumulation in the gas phase) (0.39 mM/h), and therefore the hold-up was considered insignificant. With a Henry's law constant of 8.7 · 10<sup>-9</sup> M/Pa (70°C) the critical dissolved hydrogen concentration is estimated to be 500 μM.

*C. saccharolyticus* appeared to be relatively sensitive to organic fermentation products (Table I). So far, inhibition of thermophilic growth by such compounds has only been described for *T. ethanolicus* (Wiegel and Ljungdahl, 1981). Compared to *C. saccharolyticus*, this organism is more tolerant to acetate ([Ac]<sub>crit</sub> = 450 mM), lactate ([Lac]<sub>crit</sub> = 400 mM), and especially ethanol ([Et]<sub>crit</sub> = 1.3 M) at a temperature of 65°C and a pH of 7.5. Inhibition of growth by acetate and other weak organic acids is quite common, but the level of tolerance varies tremendously among spe-



**Figure 5.** Effect of the concentration of sodium acetate on the growth rate and cell lysis. (A) Different added concentrations of sodium acetate (●) 0 mM; (○) 73 mM; (■) 143 mM; (◻) 209 mM; (▲) 272 mM. (B) Hydrogen production (as measured in the gas phase) in the presence of (●) 0 mM; (■) 145 mM; (▲) 275 mM sodium acetate. (C) Rate of change in OD (calculated from the slopes in Fig. 5A) versus sodium acetate concentrations. The line represents best fit of Eq. (6) through the data points.

cies (Lasko et al., 2000). The undissociated form of these compounds can function as uncoupling agents (Booth, 1985). However, like with *Clostridium thermoaceticum* (Wang and Wang, 1984), the ionic strength of sodium acetate was responsible for the inhibition of the hydrogen production by *C. saccharolyticus* (Table I, Fig. 4B) and



**Figure 6.** Growth of *C. saccharolyticus* on sucrose in batch culture, with continuous removal of H<sub>2</sub>. The line through the data points of biomass represents best fit of Eq. (4). Biomass (●), sucrose (○), and acetate (■).

acetic acid had no effect at slightly acidic pH (6–7). This is not surprising when taking its low pK<sub>a</sub> value of 4.75 into account.

High concentrations of sodium acetate induced cell lysis in *C. saccharolyticus* (Fig. 5). Donnison et al. (1989) already observed lysis in exponentially growing cultures of this organism after addition of 20 mM sodium acetate. Our results agree with their finding, however, in exponentially growing cultures cell lysis did not dominate until acetate concentrations exceeded 150–175 mM (Fig. 5C). Sodium chloride and potassium acetate caused the same effect, indicating that high cation concentrations were responsible for cell lysis. Similar phenomena have already been described for, among others, *Cl. thermoaceticum* (Wang and Wang, 1984) and *Bacillus subtilis* (Fan, 1970; Jolliffe et al., 1981). Studies with the latter organism revealed the release of autolysins upon addition of high salt concentrations. Inactive autolysins are distributed in the relatively acidic cell wall of Gram-positive bacteria during exponential growth (Kemper et al., 1993). It is thought that autolysins become activated once the pH of the cell wall is neutralized. One way to accomplish this is by dissipation of the proton motive force, e.g., by exposure to high external salt concentrations. Results obtained in this study imply a similar scenario in *C. saccharolyticus*, which possesses also a gram-positive-type cell wall (Hamana et al., 1996).

In summary, the fermentation of sucrose by *C. saccharolyticus* is inhibited by several parameters, making optimization of the process a complex one. For an optimal hydrogen production process it is of utmost importance that hydrogen is removed from the culture with high efficiency. Inhibition by sucrose can be avoided simply by applying a continuous-flow or a fed-batch fermentation process. As fermentation products accumulate in the culture fluid, the ionic strength might then become the dominant inhibitor (Fig. 6). This can be kept under control, e.g., by diluting the

sugar fermentation broth by recycling part of the effluent of a coupled acetate fermentation reactor.

We thank Wim Roelofsen for his technical assistance during the investigations.

## REFERENCES

- Adams MWW. 1990. The metabolism of hydrogen by extremely thermophilic, sulfur-dependent bacteria. *FEMS Microbiol Rev* 75:219–238.
- Adams MWW, Mortenson LE, Chen J-S. 1981. Hydrogenase. *Biochim Biophys Acta* 594:105–176.
- Benemann JR. 1996. Hydrogen biotechnology: progress and prospects. *Nat Biotechnol* 14:1101–1103.
- Booth IR. 1985. Regulation of cytoplasmic pH in bacteria. *Microbiol Rev* 49:359–378.
- Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing protein–dye binding. *Anal Biochem* 72:248–254.
- Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA. 1999. Utilisation of biomass for the supply of energy carriers. *Appl Microbiol Biotechnol* 52:741–755.
- Donnison AM, Brockelsby CM, Morgan HW, Daniel RM. 1989. The degradation of lignocellulosics by extremely thermophilic microorganisms. *Biotechnol Bioeng* 33:1495–1499.
- Fan DP. 1970. Cell wall binding properties of the *Bacillus subtilis* autolysin(s). *J Bacteriol* 103:488–493.
- Fiala G, Stetter KO. 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaeobacteria growing optimally at 100°C. *Arch Microbiol* 145:56–61.
- Gottschalk G. 1986. Bacterial metabolism. New York: Springer-Verlag. p 359.
- Hamana K, Hamana H, Niitsu M, Samejima K. 1996. Polyamines of thermophilic Gram-positive anaerobes belonging to the genera *Caldicellulosiruptor*, *Caloramator*, *Clostridium*, *Coprothermobacter*, *Moorella*, *Thermoanaerobacter* and *Thermoanaerobacterium*. *Microbios* 85:213–222.
- Han K, Levenspiel O. 1988. Extended Monod kinetics for substrate, product, and cell inhibition. *Biotechnol Bioeng* 32:430–437.
- Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO. 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. *Arch Microbiol* 144:324–333.
- Janssen PH, Morgan HW. 1992. Heterotrophic sulfur reduction by *Thermotoga* sp. Strain FjSS3.B1. *FEMS Microbiol Lett* 96:213–218.
- Joliffe LK, Doyle RJ, Streips UN. 1981. The energized membrane and cellular autolysis in *Bacillus subtilis*. *Cell* 25:753–763.
- Kemper MA, Urrutia MM, Beveridge TJ, Koch AL, Doyle RJ. 1993. Proton motive force regulate cell wall-associated enzymes of *Bacillus subtilis*. *J Bacteriol* 175:5690–5696.
- Kengen SWM, Stams AJM. 1994. Formation of L-alanine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus*. *Arch Microbiol* 161:168–175.
- Kengen SWM, Stams AJM, de Vos WM. 1996. Sugar metabolism of hyperthermophiles. *FEMS Microbiol Rev* 18:119–137.
- Lasko DR, Zamboni N, Sauer U. 2000. Bacterial response to acetate challenge: a comparison of tolerance among species. *Appl Microbiol Biotechnol* 54:243–247.
- Malik B, Su W-W, Wald HL, Blumentals II, Kelly RM. 1989. Growth and gas production of hyperthermophilic archaeobacterium, *Pyrococcus furiosus*. *Biotechnol Bioeng* 34:1050–1057.
- Pauss A, Andre G, Perrier M, Guiot SR. 1990. Liquid-to-gas mass transfer in anaerobic processes: inevitable transfer limitations of methane and hydrogen in the biomethanation process. *Appl Environ Microbiol* 56:1636–1644.
- Rainey FA, Donnison AM, Janssen PH, Saul D, Rodrigo A, Bergquist PL, Daniel RM, Stackebrandt E, Morgan HW. 1994. Description of *Caldicellulosiruptor saccharolyticus* gen. nov., sp. nov.: an obligately anaerobic, extremely thermophilic, cellulolytic bacterium. *FEMS Microbiol Lett* 120:263–266.
- Robinson JA. 1985. Determining microbial kinetic parameters using non-linear regression analysis. In: *Advances in microbial ecology*. New York: Plenum Press. p 61–114.
- Schäfer T, Schönheit P. 1991. Pyruvate metabolism of the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Arch Microbiol* 155:366–377.
- Schönheit P, Schäfer T. 1995. Metabolism of hyperthermophiles. *World J Microbiol Biotechnol* 11:26–57.
- Schröder C, Selig M, Schönheit P. 1994. Glucose fermentation to acetate, CO<sub>2</sub>, and H<sub>2</sub> in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden–Meyerhof pathway. *Arch Microbiol* 161:460–470.
- Trevelyan WE, Harrison JS. 1952. Studies on yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. *Biochem J* 50:298–310.
- Wang G, Wang DIC. 1984. Elucidation of growth inhibition and acetic acid production by *Clostridium thermoaceticum*. *Appl Environ Microbiol* 47:294–298.
- Wiegel J, Ljungdahl LG. 1981. *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. *Arch Microbiol* 128:343–348.