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Biohydrogen production in alkalithermophilic conditions: *Thermobrachium celere* as a case study

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ABSTRACT

In the present work the hydrogenesis in the anaerobic alkalithermophilic bacterium *Thermobrachium celere* was studied. The impact of several factors on hydrogen production during glucose fermentation was investigated in batch conditions. The optimal hydrogen production occurred at $pH_{67\ °C}$ 8.2 with phosphate buffer concentration of 50 mM. Hydrogen yield reached the highest value of 3.36 mol H_2/mol glucose when the partial pressure in the gas headspace was reduced. Supplementation of nitrogen sources and iron affected hydrogen production. Under optimized conditions, the maximum H_2 accumulation and H_2 production rate were estimated to be respectively 124.3 mmol H_2/l culture and 20.7 mmol H_2/l/h. Considering the efficient and rapid hydrogen evolution, and the ability to grow in extreme environments, *T. celere* might be a good candidate for biohydrogen production in open (non-sterile) bioprocess system.

1. Introduction

Light-independent (dark) fermentation by anaerobic bacteria is a promising biological process for converting carbohydrate-rich substrates to hydrogen. Compared to photofermentation it has several advantages such as higher hydrogen production rate and capability to convert organic wastes to more valuable energy sources (Levin et al., 2004). Theoretically, the stoichiometric reaction of fermentative biohydrogenation process anticipates a maximum yield of 4 mol H_2/mol glucose consumed when hexose is oxidized to acetate and CO_2 (Thauer et al., 1977). However, this yield can be achieved exclusively when the only volatile fermentation product is acetate. In practice, hydrogen and acetate are not the only fermentation end products since more reduced molecules may also be produced lowering the hydrogen yield.

Mesophilic bacteria normally achieve a H_2 yield in the order of 2 mol H_2/mol glucose due to the production of different by-products and thermodynamic limitation associated with NADH as the electron donor for H_2 reduction. Hydrogen production from thermophilic (50–55 °C) or extreme thermophilic (55–80 °C) bacteria has however been reported to generate higher yields compared to those of mesophiles (Chou et al., 2008). Conversion of carbon sources to hydrogen is more efficient at high temperatures since the reaction is thermodynamically favorable (Kengen et al., 2008), allowing a lower variety of fermentation end products and a yield near the theoretical limit (Zeidan and van Niel, 2010; Schröder et al., 1994). This poses a clear advantage when thermophiles are used for hydrogen production.

Hydrogen partial pressure (P_{H_2}) in the gas phase is another factor affecting hydrogen yield. When the concentration of H_2 in the headspace increases, H_2 synthesis becomes thermodynamically unfavorable and the metabolism is shifted towards the production of more reduced end products (Levin et al., 2004). In particular, the equilibrium of the reaction that involves electron transfer from NADH to ferrodoxin by NADH ferrodoxin:oxidoreductase (NFOR) is strongly affected by the H_2 partial pressure (Mandal et al., 2006). Therefore, only at low P_{H_2} value NADH can be oxidized to NAD^+ through H_2 synthesis boosting the yield up to the maximum 4 mol H_2/mol glucose. Previous studies have shown (Nguyen et al., 2010; van Niel et al., 2002, 2003) that high H_2 yields can be achieved when fermentative hydrogen production is operated at thermophilic temperatures and at low hydrogen partial pressure.

Another advantage of operating fermentation at high temperature is the inhibition of mesophilic organisms, like H_2 consuming methanogens, that can compromise the hydrogen production (Chou et al., 2008). Prevention of contamination could be even more effective if thermophilic conditions were associated to other extreme parameters, such as pH or salinity. Thus, polyextremophiles can be promising organisms to be operated in open system bioreactors as the sterilization step can be avoided reducing the operational costs.

Koskinen et al. (2008) showed that a thermophilic mixed culture producing hydrogen at high yield was dominated by bacterial

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strains closely affiliated with *Thermobrachium celere*, suggesting the high potential for fermentative hydrogen evolution by this species. *T. celere* is an anaerobic alkalithermophilic bacterium with an optimal growth temperature of 67 °C, an optimal $pH_{67\ °C}$ of 8.2 and a doubling time reported as low as 10 min (Engle et al., 1996).

In this study the characterization of hydrogen production in the alkalithermophile *T. celere* is reported. The influences of chemophysical parameters on hydrogen production as well as the metabolite formation pattern during glucose fermentation were investigated in batch conditions. Thus far, no data seems to be available on this bacterial species regarding the hydrogen production. Therefore, in the present work we provide the first insight into the hydrogen metabolism in *T. celere*.

2. Methods

2.1. Medium and culture conditions

T. celere (DSMZ 8682^T from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was grown anaerobically in a modified version of the ATCC 2072 medium containing (g/l): KH_2PO_4 0.64; Na_2HPO_4 1.65; KCl 1; $(NH_4)_2SO_4$ 0.5; NH_4Cl 0.5; $MgCl_2{\cdot}6H_20$ 0.1; $CaCl_2{\cdot}6H_20$ 0.11; Cystein-HCl 0.13; $Na_2S{\cdot}9H_20$ 0.13; yeast extract 2; resazurin (redox indicator) 0.001. After sterilization glucose was added as substrate in concentration of 25 or 50 mM as well as 10 ml/l of vitamin solution and 10 ml/l trace element solution (ATCC medium No. 2072, American Type Culture Collection). All the chemicals used were of analytical grade. The pH of the medium was adjusted to 8.2 with 3 M NaOH at 67 °C. Aliquots of the medium were dispensed anaerobically into 120 ml serum bottles and sterilized by autoclaving for 15 min at 121 °C. After the inoculum (5% v/v) was added the serum bottles were incubated for 24 h at 67 °C (150 rpm).

2.2. Experimental procedures

The effect of initial pH was studied cultivating bacteria in 120 ml serum bottles containing 50 ml of medium with 25 mM of glucose. The $pH_{67\ °C}$ of the medium was adjusted from 6.2 to 9.2 with 3 M HCl or 3 M NaOH. Later on, the initial $pH_{67\ °C}$ was adjusted to 8.2. The effect of phosphate buffer was tested in the same conditions using concentrations ranging from 0 to 50 mM. The subsequent experiments were performed with 50 mM phosphate buffer. The effect of hydrogen partial pressure was investigated using different culture volumes (from 10 to 90 ml) in 120 ml serum bottles with 25 mM of glucose as substrate. To evaluate the influence of different nitrogen sources, the effect of yeast extract and tryptone was tested in 120 ml serum bottles containing 20 ml of medium supplemented with 25 or 50 mM of glucose. The effect of iron (Fe^{2+}) on H_2 production was studied using $FeSO_4$ concentrations ranging from 1 to 200 mg/l using 120 ml serum bottles containing 20 ml of medium supplemented with 50 mM of glucose. The kinetics of hydrogen production was tested under optimal medium and conditions. Negative controls without added substrate were included in all experiments.

2.3. Analytical methods and calculations

Cell concentrations were determined by measuring the absorbance spectrophotometrically at 600 nm with an Ultrospec 500 pro spectrophotometer (Amersham Biosciences, Munich, Germany). Cell dry weight (CDW) was determined by centrifuging the culture broths. For this, 20 ml aliquots of the culture were transferred into dried, pre-weighed 50 mL Falcon tubes and centrifuged at 5000*g* for 15 min. Cell pellets were washed twice with saline by centrifugation and dried at 70 °C to a constant weight. The relation between OD and CDW of *T. celere* for growth on glucose was found to be CDW (g/l) = $0.208 \times OD_{600} + 0.071$ ($R^2 = 0.99$). End point optical density was not measured due to flocculation and lysis of the biomass. The pH of the cultures was measured with a pH330i pH meter and a Sentix 41 pH-electrode (WTW, Weilheim, Germany), according to the methods described by Engle et al. (1996).

The amount of biogas produced was measured using a syringe method. The composition of biogas was sampled by using a gas-tight syringe (0.3 mL injection volume) and analyzed with a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and a column packed with Porapak N (80/100 mesh). N_2 was used as a carrier gas and the temperatures of the injector, column and detector were 110, 80 and 110 °C, respectively. Hydrogen gas production was calculated from the headspace gas sample and the total volume of biogas produced at each time interval according to the mass balance equation (Eq. (1))

$$V_{H_2,t} = V_{H_2,t-1} + C_{H_2,t}(V_{g,t} - V_{g,t-1}) + V_{Head}(C_{H_2,t} - C_{H_2,t-1}) \quad (1)$$

where $V_{H_2,t}$ is the cumulative H_2 gas volume at time t, $V_{H_2,t-1}$ is the cumulative H_2 gas volume at time $t-1$, $V_{g,t}$ is the cumulative total gas volume at time t, $V_{g,t-1}$ is the cumulative total gas volume at time $t-1$, V_{Head} is the headspace volume in serum bottles, $C_{H_2,t}$ is the H_2 percentage of biogas in headspace at time t, and $C_{H_2,t-1}$ is the H_2 percentage of biogas in headspace at time $t-1$. Molar H_2 and CO_2 were calculated using the ideal gas law.

Soluble end products (VFA and ethanol) and glucose in the supernatant were analyzed by high liquid performance chromatography (HPLC) with an LC-20AC prominence liquid chromatograph equipped with an RID-10A refractive index detector, DGU-20A5 prominence degasser and a CBM-20A prominence communications bus module (Shimadzu, Kyoto, Japan). The column was a 30 cm Shodex® 201 Sugar SH1011 column. 0.01 N H_2SO_4 was used as mobile phase at a flow rate of 0.900 ml/min.

Carbon balance was calculated from the total amount of carbon-containing products formed (in C-mol) and the amount of sugar consumed (in C-mol). Electron balance was calculated after multiplying the amount of each metabolite and the sugar by the corresponding degree of reduction (in mol electrons per C-mol) (Zeidan and van Niel, 2010). Metabolite yields as well as carbon and electron balances were calculated by subtracting the background metabolite production in control cultivations (i.e. cultivation without the substrate) from the results.

3. Results and discussion

3.1. Effect of initial pH on H_2 production

To investigate the role of the pH on the hydrogen production, initial $pH^{67\ °C}$ levels ranging from 6.2 to 9.2 with 0.5 increments were tested in this study using 16 mM of phosphate buffer as buffering agent. The results (Fig. 1) showed that hydrogen accumulation in the headspace steadily increased with the $pH^{67\ °C}$ values from 6.2 to 8.2. Further increments in the initial pH value corresponded to a decrease of hydrogen production. At 9.2 the growth was completely inhibited and no hydrogen was detected. The highest hydrogen production of 33.2 mmol H_2/l culture was observed at the initial $pH_{67\ °C}$ 8.2.

Fig. 1 shows the VFA and ethanol production at various initial pH levels. At every pH tested the main soluble end product was acetate. Acetate concentration increased progressively at $pH_{67\ °C}$ values from 6.2 to 8.2 reaching 14.3 mM and started decreasing at 8.7 following the same pattern of hydrogen accumulation.