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Hydrogen production from glycerol using halophilic fermentative bacteria

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ABSTRACT

Glycerol-based hydrogen production by the halophilic bacteria *Halanaerobium saccharolyticum* subspecies *saccharolyticum* and *senegalensis* was studied as batch experiments. The main metabolites of glycerol fermentation of both strains were hydrogen, carbon dioxide, and acetate. Subspecies *saccharolyticum* also produced 1,3-propanediol (1,3-PD), butyrate, and ethanol. The highest hydrogen yields were achieved with 2.5 g/l glycerol and 150 g/l salt at pH 7.4 (subsp. *saccharolyticum*, yield 0.6 mol/mol glycerol) and at pH 7.0 (subsp. *senegalensis*, yield 1.6 mol/mol glycerol). The hydrogen yield of subsp. *senegalensis* has potential for practical applications after scale-up and bioprocess optimizations and metabolic engineering after genome-wide sequencing could be applied to improve the yield of subsp. *saccharolyticum*.

1. Introduction

Halophiles are microorganisms that require high salt concentration for growth. Moderate halophiles achieve optimal growth at salt concentrations from 0.5 M to 2.5 M while extreme halophiles grow optimally in salt concentrations over 2.5 M (up to saturation) (Ventosa et al., 1998). Halophilic anaerobic fermentative bacteria form the order Halanaerobiales and cope with the extreme living conditions by balancing the salt concentration of the environment with high cytoplasmic ion (mostly K^+ and Cl^-) concentrations (Oren, 2006, 2008). Another strategy for surviving in hypersaline environment includes intracellular accumulation of organic solutes e.g. glycerol (Roberts, 2005).

Glycerol plays an important role in hypersaline environments where it is mainly produced by green algae of the genus *Dunaliella*, which are typically found in hypersaline environments worldwide (Ben-Amotz and Avron, 1973; Oren, 1995). Some Halanaerobiales are capable of metabolizing glycerol and producing a variety of end products (Oren, 1995). Glycerol is produced as a by-product of the biodiesel industry and might be substrate for biotechnological applications; however, industrial glycerol often contains heavy metals and salts which can be inhibitory to microorganisms (Johnson and Taconi, 2007). Such contaminants might be less of a problem for halophilic bacteria since many have been reported to be heavy metal resistant, and high salt concentrations cause no problems (Nieto et al., 1989).

Halophilic microorganisms might also be advantageous because hypersalinity suppresses growth of most other organisms and therefore, sterilization costs can be reduced. Biotechnological applications of halophilic archaea include the production of bacteriorhodopsin, polymers, enzymes, and compatible solutes (Ventosa and Nieto, 1995). While much of the interest in halophilic bacteria has been focused on their enzymes, possible compatible solutes, and biodegradation of residues and wastes (Kapdan and Erten, 2007; Ventosa and Nieto, 1995; Dan et al., 2003), fermentative bacteria might also produce useful fermentation products such as hydrogen.

Fermentation of glycerol and hydrogen production by the Gram negative, anaerobic bacteria *Halanaerobium saccharolyticum* and *H. lacusrosei* isolated from the sediments of hypersaline lakes have already been described (Cayol et al., 1994, 1995; Zhilina et al., 1992). Since hydrogen utilizing methane producers are rarely found in hypersaline environments (Oren, 2001, 2002), hydrogen yields should not be impacted by hydrogen consuming bacteria in such environments.

In this article, a detailed study on hydrogen production and end-metabolite formation in the glycerol fermentation of *H. saccharolyticum* subspecies *saccharolyticum* and *senegalensis* is presented. *Escherichia coli* and *Clostridium butyricum*, both non-halophilic and known hydrogen producers, were used as positive control strains for hydrogen production and as reference strains for comparing the metabolites, yields, and glycerol utilization capabilities.

2. Methods

2.1. Chemicals

Unless otherwise stated, chemicals were purchased from Sigma–Aldrich Co (St. Louis, MO, USA). Bacto tryptone was

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purchased from Becton Dickinson & Co (NJ, USA), yeast extract from Scharlau Chemie (Barcelona, Spain), sodium chloride (NaCl) and ammonium chloride (NH_4Cl) from VWR International (West Chester, PA, USA). Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium molybdate (Na_2MoO_4) and sodium acetate ($C_2H_3NaO_2$) were from Mallinckrodt Baker (Phillipsburg, NJ, USA), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), potassium chloride (KCl), nicotinic acid, d-Ca-pantothenate, p-aminobenzoic acid, potassium hydroxide (KOH), and the trace element chemicals except for sodium molybdate were from Merck & Co (Whitehouse Station, NJ, USA).

2.2. Strains

H. saccharolyticum subsp. *saccharolyticum* strain DSM 6641, *H. saccharolyticum* subsp. *senegalensis* strain DSM 7379^T, and *C. butyricum* strain DSM 2478 were from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *E. coli* strain BW25113 was obtained from The Coli Genetic Stock Center (CGSC, New Haven, CT, USA).

2.3. Culture conditions and experimental procedure

The pure cultures of halophilic bacterial strains were grown in HM100 medium (NH_4Cl 1.0 g/l, K_2HPO_4 0.3 g/l, KH_2PO_4 0.3 g/l, $MgCl_2{\cdot}6H_2O$ 2.0 g/l, $CaCl_2{\cdot}2H_2O$ 0.2 g/l, KCl 4.0 g/l, Na-acetate$\cdot 3H_2O$ 1.0 g/l, Bacto tryptone 1.0 g/l, NaCl 100 g/l, cysteine–HCl 0.5 g/l, resazurin 0.002 g/l) supplemented with trace elements (HCl 0.077 mM, $FeCl_2{\cdot}4H_2O$ 1.50 mg/l, $ZnCl_2$ 0.07 mg/l, $MnCl_2{\cdot}4H_2O$ 0.10 mg/l, H_3BO_3 6.00 μg/l, $COCl_2{\cdot}6H_2O$ 0.19 mg/l, $CuCl_2{\cdot}2H_2O$ 2.00 μg/l, $NiCl_2{\cdot}6H_2O$ 24.00 μg/l, $Na_2MoO_4{\cdot}2H_2O$ 36.00 μg/l) and vitamins (biotin 20.0 μg/l, folic acid 20.0 μg/l, pyridoxine–HCl 0.10 mg/l, thiamine–HCl$\cdot 2H_2O$ 50.00 μg/l, riboflavin 50.00 μg/l, nicotinic acid 50.00 μg/l, d-Ca-pantothenate 50.00 μg/l, vitamin B_{12} 5.00 μg/l, p-aminobenzoic acid 50.00 μg/l, lipoic acid 50.00 μg/l). Potassium hydroxide was used for pH adjustments. The pure cultures of *C. butyricum* and *E. coli* were grown in 2×YT medium (16 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl; pH was adjusted with KOH). Medium for *C. butyricum* was also supplied with vitamins described above in case of halophilic medium. The slower growing halophiles were grown for 72 h and the faster growing *E. coli* and *C. butyricum* for 48 h in sealed glass tubes at 37 °C (150 rpm) under nitrogen atmosphere. The experiments were done in duplicate using sterile techniques. Negative controls without added substrate were included in all experiments.

For studies on the use of glycerol and glucose as substrates, the halophilic strains were grown in 10 ml of medium containing initial substrate concentrations of 2.5, 5, 10, and 20 g/l at 100 g/l NaCl. The initial pH was 7 (Cayol et al., 1994; Zhilina et al., 1992). For the study of the effect of yeast extract, the halophilic strains were grown in 10 ml of medium containing 5 g/l glycerol and yeast extract at concentrations of 0, 0.5, 1, and 10 g/l. The NaCl content in experiment was 100 g/l and the initial pH value was 7. The pH was not adjusted during the experiments. *E. coli* and *C. butyricum* were grown in medium containing glycerol at concentrations of 5, 10, and 20 g/l and at 10 g/l NaCl. The pH was 6.6 (Murarka et al., 2008; Saint-Amans et al., 2001).

The optimal pH, salt and glycerol concentrations for halophilic hydrogen production were determined in 10 ml of media. For the study of the effect of salt concentration on halophilic glycerol metabolism, the halophilic strains were grown in NaCl concentrations of 50 (0.85 M), 100 (1.7 M), 150 (2.6 M), and 200 g/l (3.4 M), at pH 7 and 5 g/l glycerol. These salt concentrations tested were chosen, according to literature, around the optimal salt content for the growth of the strains (Cayol et al., 1994; Zhilina et al., 1992). For the study on the effect of pH on glycerol fermentation and hydrogen production, the halophilic strains were grown in medium with initial pH values of 6.6, 7.0, 7.4, 7.8, and 8.2 (with range of variation 0.05), at 100 g/l NaCl and 5 g/l glycerol. The pH values were chosen near the values where halophilic strains achieve optimal growth (Cayol et al., 1994; Zhilina et al., 1992). The effect of pH was also determined with *E. coli* and *C. butyricum*, which were grown in the pH values of 6.6, 7, and 7.4, at 10 g/l NaCl and 5 g/l glycerol. The pH was not adjusted during the experiments.

Optimal conditions were chosen for further analyses based on the highest hydrogen yields (mol H_2/mol glycerol consumed). Hydrogen production was evaluated in batch experiments with 50 ml of medium under the optimal conditions observed in previous experiments. Control cultivations were prepared without glycerol.

2.4. Analyses

Upon termination of the cultivations, the pH was measured directly from un-filtered end-point batch media with a pH330i pH-meter and a Sentix 41 pH-electrode (WTW, Weilheim, Germany). Optical density, OD_{600}, was determined from the end-point batch cultivations with an Ultrospec 500 pro spectrophotometer (Amersham Biosciences, Munich, Germany). The optical density of *H. saccharolyticum* subsp. *senegalensis* cultures was not measured due to flocculation of the biomass. Hydrogen, carbon dioxide, and methane were analyzed with a Shimadzu GC-2014 gas chromatograph equipped with a thermal conductivity detector. The temperatures of column and detector oven were 80 °C and 110 °C, respectively. Nitrogen was used as a carrier gas. Gas samples were analyzed in duplicate.

Liquid end products were analyzed using high liquid performance chromatography (HPLC). Samples of bacterial cultures were centrifuged at 11 000 g for 5 min, and the supernatant was filtered through a polycarbonate filter (Chromafil® 194 195 PET-45/25, Macherey-Nagel, Düren, Germany). The samples were analyzed for glucose, succinate, lactate, glycerol, formate, acetate, 1,3-propanediol (1,3-PD), propionate, ethanol, and butyrate at a column temperature of 54 °C with an LC-20AC prominence liquid chromatograph equipped with an RID-10A refractive index detector, DGU-20A5 prominence degasser, CBM-20A prominence communications bus module, and SIL-20AC 200 prominence autosampler (Shimadzu, Kyoto, Japan). The column was a 30-cm Shodex® 201 Sugar SH1011 column (Showa Denko, Tokyo, Japan). As an eluent, 0.01 N H_2SO_4 was used at a flow rate of 0.600 ml/min. The metabolites were identified based on retention times and the concentrations (mM) and yields (mol product/mol substrate consumed) were calculated as average of duplicate experiments with standard deviations. The concentrations of the liquid products are reported as concentrations in the liquid phase and the concentrations of the gaseous products as concentrations in the gaseous phase. Unless otherwise mentioned, the background metabolite production in control cultivations (i.e. cultivation without the substrate) was subtracted from the results.

3. Results and discussion

3.1. Glycerol as a substrate

The concentrations of the main end-metabolites produced by the halophilic and the non-halophilic strains in different glycerol concentrations are shown in Fig. 1. The main end-metabolites of *H. saccharolyticum* subsp. *saccharolyticum* were hydrogen (H_2), carbon dioxide (CO_2), acetate, and 1,3-PD in addition to minor amounts of butyrate and ethanol. *H. saccharolyticum* subsp. *senegalensis* converted glycerol mainly into H_2, CO_2, and acetate. Additionally, a