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Glycerol fermentation to hydrogen by *Thermotoga maritima*: Proposed pathway and bioenergetic considerations

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ABSTRACT

The production of biohydrogen from glycerol, by the hyperthermophilic bacterium *Thermotoga maritima* DSM 3109, was investigated in batch and chemostat systems. *T. maritima* converted glycerol to mainly acetate, CO_2 and H_2. Maximal hydrogen yields of 2.84 and 2.41 hydrogen per glycerol were observed for batch and chemostat cultivations, respectively. For batch cultivations: i) hydrogen production rates decreased with increasing initial glycerol concentration, ii) growth and hydrogen production was optimal in the pH range of 7–7.5, and iii) a yeast extract concentration of 2 g/l led to optimal hydrogen production. Stable growth could be maintained in a chemostat, however, when dilution rates exceeded 0.025 h^{-1} glycerol conversion was incomplete. A detailed overview of the catabolic pathway involved in glycerol fermentation to hydrogen by *T. maritima* is given. Based on comparative genomics the ability to grow on glycerol can be considered as a general trait of *Thermotoga* species. The exceptional bioenergetics of hydrogen formation from glycerol is discussed.

1. Introduction

Hydrogen gas (H_2) is considered an attractive alternative to fossil fuels, as it burns cleanly, without emitting carbon dioxide (CO_2) or any other environmental pollutants [1]. H_2 possesses the highest energy content per unit of weight compared to other fuels, and it can be used in energy-efficient hydrogen fuel cells [2]. However, nearly 96% of the total current H_2 production, by catalytic steam reforming of natural gas, coal gasification or the partial oxidation of refinery oil, is still derived from fossil fuels. Since these processes are not based on renewable resources and still cause a net increase of atmospheric CO_2, they are not considered sustainable [3,4]. To overcome the use of fossil hydrocarbons as sources for H_2 production, alternative methods, like electrolysis, thermal decomposition of water and biological methods, are preferred. The electro- and thermo-chemical means are very energy inefficient. Moreover, they still depend on fossil fuels for the generation of electricity and heat [5]. Biological hydrogen (biohydrogen) production by bacteria, on the other hand, is far more promising due to its potential as an inexhaustible, low-cost and environmentally friendly process, especially when it is generated from a variety of renewable resources [6,7]. Biohydrogen is produced either by biophotolysis, microbial electrolysis, photo-fermentation, using light-dependent organisms, or by dark fermentation [8]. Biohydrogen production by dark fermentation is an anaerobic process, involving heterotrophic fermentative bacteria or archaea that convert biomass or biomass-derived hydrocarbons mainly to H_2 and acetate. To enhance the economy of H_2 production by dark fermentation it is important to explore suitable biomass substrates which can be utilized by a broad range of H_2 producing microorganisms.

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Recently many research efforts have been devoted to microbial conversion of low-priced industrial and agricultural waste into bioenergy [9–12]. One of these industrial wastes concerns crude glycerol [12–14]. Crude glycerol is an inevitable by-product of the production of biodiesel; about 10 kg crude glycerol, containing 50–60% pure glycerol, is produced for every 100 kg of biodiesel [15]. In recent years, the accelerated growth of the biodiesel industry has generated a surplus of glycerol, that resulted in a 10-fold decrease in crude glycerol prices. Furthermore, this has generated environmental concern associated with glycerol disposals [14]. As a result, glycerol has gone from being a chemical commodity to a chemical waste in less than a decade. Its availability, low price and its potential to mitigate possible environmental hazards make glycerol an attractive carbon source for industrial microbiology including the dark fermentation processes.

Yet another advantage is that fuels and reduced chemicals can be produced from glycerol at yields higher than those obtained from common sugars like glucose and xylose [14]. This is due to its highly reduced redox state of carbon, the degree of reduction per carbon for glucose and xylose is 4 compared to 4.67 for glycerol [16].

Until recently, the fermentative metabolism of glycerol had been reported in species of the genera *Klebsiella, Citrobacter*, *Enterobacter*, *Clostridium, Lactobacillus, Bacillus, and Anaerobiospirillum* [13,14]. However, the potential for using these mesophilic organisms for H_2 production in dark fermentation is limited due to the low yield. In previous studies converting pure glycerol or glycerol-containing wastes [13,17,18] the maximum H_2 yield obtained was ~1 mol H_2 per mol of glycerol, concomitant with the production of ~1 mol of ethanol per mol of glycerol. Moreover, mesophilic microorganisms often produce reduced end-products such as diols and lactic acid, at the expense of H_2 [13,19]. Therefore, for maximal H_2 production, oxidation of glycerol to acetic acid is required.

In light of yield optimization of H_2 from biomass, extreme thermophilic anaerobic bacteria are preferred. Their yields are reported to be approximately 83–100% of the maximum theoretical value of 4 mol hydrogen/mol glucose, in contrast to the mesophilic facultative anaerobes which show a H_2 yield of less than 2 [20]. Furthermore, thermophilic H_2 production benefits from some general advantages of performing processes at elevated temperatures, like a lower viscosity, better mixing, less risk of contamination, higher reaction rates and no need for reactor cooling [21]. Among the thermophiles, the order of the *Thermotogales* is characterized by the ability of its members to utilize a wide variety of carbohydrates [22] and to ferment sugars predominantly to acetate, CO_2, and H_2 [23,24].

However, in literature some contradiction exists concerning the ability of *Thermotoga* species to convert glycerol. Previous studies reported that *Thermotoga maritima* contains the coding sequences for a complete pathway for both glycerol uptake and conversion [25]. A positive signal indicating oxidation of glycerol by *Thermotoga neapolitana* was found in a microplate assay [26]. Ngo et al. describes hydrogen production by *T. neapolitana* on biodiesel waste with a maximal yield of 2.73 mol H_2/mol glycerol consumed [27]. However, Eriksen et al. could not observe glycerol conversion by *T. maritima, T. neapolitana,* or *Thermotoga elfii* [28]. These, opposing results prompted us to reinvestigate the ability of *Thermotoga* species to use glycerol. Our preliminary data showed that *T. neapolitana*, but also *T. maritima* were able to form hydrogen from glycerol [29].

Here, biohydrogen production from glycerol by *T. maritima* was investigated in detail. Optimum growth parameters and cultivation conditions were determined. A putative glycerol catabolic pathway leading to hydrogen is presented, and the unusual thermodynamics and biochemistry of high yield hydrogen formation from glycerol are discussed.

2. Material and methods

2.1. Organisms and growth conditions

T. maritima strain DSM 3109 [22] and *Thermotoga neapolitana* strain DSM 4359 [30] were obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen and cultivated in M3 medium. M3 medium, which was based on M2 medium [31], consisted of (amounts are in grams per liter of deionized water): 1.5 g KH_2PO_4; 2.4 g $Na_2HPO_4 \cdot 2H_2O$; 0.5 g NH_4Cl; 0.2 g $MgCl_2 \cdot 6H_2O$; 2.0 mg $NiCl_2 \cdot 6H_2O$; NaCl, 2.7% (w/v) for *T. maritima* and 2.0% (w/v) for *T. neapolitana*; 11.9 g HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2 ethanesulphonic acid); 2 g yeast extract (YE); 15 mL trace element solution (DSM-TES, see DSMZ medium 141, complemented with Na_2WO_4 3.00 mg/L); 1.0 mL of vitamin solution (Biotin 2 mg, Nicotinamide 20 mg, *p*-Aminobenzoic acid 10 mg, Thiamine (Vit.B_1) 20 mg, Pantothenic acid 10 mg, Pyridoxamine 50 mg, Cyanocobalamin and Riboflavin 10 mg); 1.0 g/L of cysteine hydrochloride as reducing agent and 1 mg resazurin, which was used as a redox indicator. Anaerobic conditions were achieved by flushing the headspace of the serum bottles with N_2 gas. The starting pH of the medium was adjusted to pH 6.9 for *T. maritima* and pH 7.3 for *T. neapolitana* with 10 mM NaOH.

Batch cultivations were performed in 120- and 240-mL serum bottles with a working volume of 25 ml or 50 mL, at a constant temperature of 80 °C and shaking at 200 rpm. Cultures were inoculated with a 10% (v/v) pre-culture. The effect of the glycerol concentration (2.5–40 g/L) on the fermentation performance was tested for both *T. maritima* and *T. neapolitana*. Optimal growth parameters (pH, YE concentration) for glycerol (2.5 g/L) conversion by *T. maritima* were investigated for the pH range of 4.9–9.2 and YE concentration range of 0–4 g/L.

Chemostat cultivations of *T. maritima* were performed in a 2-l jacketed bioreactor (Applikon) with a working volume of 1 L. Fermentations were run at 80 °C, using a stirring speed of 300 rpm and pH was controlled at 7.0 by automatic addition of 2 N NaOH. The broth was continuously sparged with N_2 gas (4 NL/h). To prevent the loss of volatile end products via the gas phase, off-gas was led through a water cooled condenser (4 °C). Cultivations were performed in the M3 medium without HEPES, using a glycerol concentration of 2.5 g/L and a YE concentration of 2 g/L. The medium was inoculated with an exponentially growing pre-culture (5% (v/v)). During the batch start-up phase the broth was not sparged and the gas outlet was closed, mimicking the closed bottle setup. Fermentation performance was investigated during growth at different dilution rates (0.017, 0.025, 0.036 and 0.050 h^{-1}). The system was assumed to be in steady state when H_2 and CO_2