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Biohydrogen production from glycerol using *Thermotoga* spp.

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Abstract

Given the highly reduced state of carbon in glycerol and its availability as a substantial byproduct of biodiesel production, glycerol is of special interest for sustainable biofuel production. Glycerol was used as a substrate for biohydrogen production using the hyperthermophilic bacterium, *Thermotoga maritima* and *Thermotoga neapolitana*. Both species metabolized glycerol to mainly acetate and hydrogen. At glycerol concentrations of 2.5 g/L, hydrogen was produced with a yield of 2.75 and 2.65 mol H₂/mol glycerol consumed by *T. maritima* and *T. neapolitana* respectively. Additionally, the effect of initial pH (ranging between pH 5.0-8.5) and yeast extract concentrations (0.5, 1, 2, 4 g/L) on glycerol fermentation by *T. neapolitana* was investigated in batch systems. An initial pH value of around 7 was optimal for hydrogen production by *T. neapolitana*. Lower concentration of yeast extract resulted in a lower H₂ production, however increasing the concentration from 2 to 4 g/L did not affect H₂ production.

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1. Introduction

An increasing societal interest and an expected increase in demand for hydrogen gas (H_2) as fuel for fuel cells, requires the usage of renewable resources (wind, sun, hydropower, geothermics and biomass) for H_2 production in order to avoid environmental impacts, since presently hydrogen is produced directly or indirectly from fossil fuels. Biological H_2 production from biomass can be done using anaerobic fermentative microorganisms in a process generally designated as 'dark fermentation'.

To enhance the yield and economy of biological hydrogen production by dark fermentation it is important to explore suitable biomass substrates which can be utilized by a broad range of hydrogen producing microorganisms. Several substrates, mainly carbohydrate sources such as glucose [1], xylose [2], sucrose [3] and starch [4] have been tested in an attempt to maximize biohydrogen production by dark fermentation. However these carbon sources are very costly. Alternatively, the feasibility of using organic wastes or waste waters [5], lignocellulosic agricultural residues, starch based materials and tofu-processing [6-8] has also been widely studied for biohydrogen production. Recently glycerol waste from the biodiesel industry has emerged as a promising substrate for bioconversions [9]. The world biodiesel production is increasing; in 2010 the total annual production capacity in the US and EU was 6.9 million tonnes and it is expected to be doubled in 2012 [10]. Since ~ 1 kg glycerol waste is generated for every 10 kg of biodiesel produced, it is evident that a vast amount of glycerol will be produced, which currently has little economic value. But, since glycerol is a more reduced compound compared to substrates like glucose or xylose, glycerol generates more NAD(P)H and potentially more hydrogen during its catabolism [11]. In this regard, glycerol is considered to be an excellent carbon source for biohydrogen production via anaerobic fermentation.

Theoretically a maximum of 3 mol H_2 can be produced per mol of glycerol when acetate is the single fermentation end product. However, in previous studies it has been observed that converting pure glycerol or glycerol-containing wastes [12-14] when using mesophilic bacteria leads to a maximum H_2 yield of around 1 mol H_2 per mol of glycerol concomitant with the production of ~ 1 mol of ethanol per mol of glycerol. Even it is more common using mesophilic microorganisms to have reduced end-products such as diols and lactic acid, yet lower H_2 generation [15]. Therefore, for maximal H_2 production, oxidation of glycerol to acetic acid is preferred. In general, H_2 production at elevated temperatures is thermodynamically more favorable [16]. 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 [17]. The hyperthermophilic bacteria *Thermotoga maritima* and *Thermotoga neapolitana* have been shown to be good candidates for biological H_2 production; when grown on glucose their H_2 yields approach the theoretical maximum yield of 4 mol H_2 mol⁻¹ glucose [1, 18].

However, in literature some controversy exists concerning the ability of *Thermotoga* species to convert glycerol. Previous studies reported that *T. maritima* contains the coding sequences for a complete pathway for both glycerol uptake and conversion [19], and a positive signal indicating oxidation of glycerol by *T. neapolitana* was found in a microplate assay [20]. Ngo et al. describes hydrogen production by *T. neapolitana* on biodiesel waste with a yield of 2.73 mol H_2 /mol glycerol consumed [21]. However, Eriksen et al. [22] could not observe glycerol conversion by *T. maritima*, *T. neapolitana*, or *T. elfii*. Therefore it is not clear to what extent *Thermotoga* species are able to utilize glycerol for growth and H_2 synthesis.

The main aim of this study was to investigate biohydrogen production from glycerol by *T. maritima* and *T. neapolitana* and to determine the optimum growth parameters such as optimal medium, initial pH and yeast extract concentrations.

2. Material and Methods

2.1. Microbial species and Culturing

Two hyperthermophilic bacteria, viz. *Thermotoga maritima* strain DSM 3109 and *Thermotoga neapolitana* strain DSM 4359, were analyzed for H₂ production.

Anaerobic batch cultivations were performed in 120- and 240-mL serum bottles with a working volume of 25 mL or 50 mL, at constant temperature of 80°C and shaking at 200 rpm. Media were inoculated with a 10% (v/v) pre-culture. Growth on glucose (2.5 g/L) and glycerol (5 g/L) by *T. maritima* was tested on three different types of media, indicated as medium A1 [23], A2 [23], and M3. M3 media was a modified M2 [21] medium consisting of (amounts are in grams per liter of deionized water): 1.5 g KH₂PO₄; 2.4 g Na₂HPO₄·2H₂O; 0.5 g NH₄Cl; 0.2 g MgCl₂·6H₂O; 2.0 mg NiCl₂·6H₂O; 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 Na₂WO₄ 3.00 mg/L); 1.0 mL of vitamin solution (Biotin 2 mg, Nicotinamide 20 mg, *p*-Aminobenzoic acid 10 mg, Thiamine (Vit.B₁) 20 mg, Pantothenic acid 10 mg, Pyridoxamine 50 mg, Cyanocobalamin and Riboflavin 10 mg [24]); 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 pure N₂ for 5 min. Growth on glucose (2.5 g/L) and glycerol (5g/L) by *T. neapolitana* was performed on M3 medium. The NaCl concentration used was 2.7% (w/v) for *T. maritima* and 2.0% (w/v) for *T. neapolitana*. 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.

The optimal growth parameters (pH, YE concentration) for glycerol (2.5 g/L) conversion by *T. neapolitana* was investigated on M3 medium for the pH range of 5-8.5 (YE, 2g/L) and YE concentration range 0- 4 g/L (pH7).

2.2. Analytical methods

Gas samples were taken from the headspace of the serum bottles using syringes and analyzed by GC, equipped with a Poraplot Q column. During batch culturing liquid samples were taken (1 ml) and growth was determined by measuring the optical density (OD₆₀₀). After centrifugation, residual substrate and organic acids in the supernatant were analyzed by HPLC, using an Shodex RSpak KC-811 ion exclusion chromatography column operating at 80°C with a eluent of 3mM H₂SO₄ (0.8 ml/min).

3. Results and Discussions

3.1. Media selection

Growth of *T. maritima* on glycerol (5 g/L) was tested on three different media, A1, A2 and M3. Under similar cultivation conditions a H₂ production of 41, 58, 65 mmol/L was observed for media A1, A2 and M3, respectively. Based on the enhanced H₂ production M3 was selected as the preferred medium, and was used in further experiments.

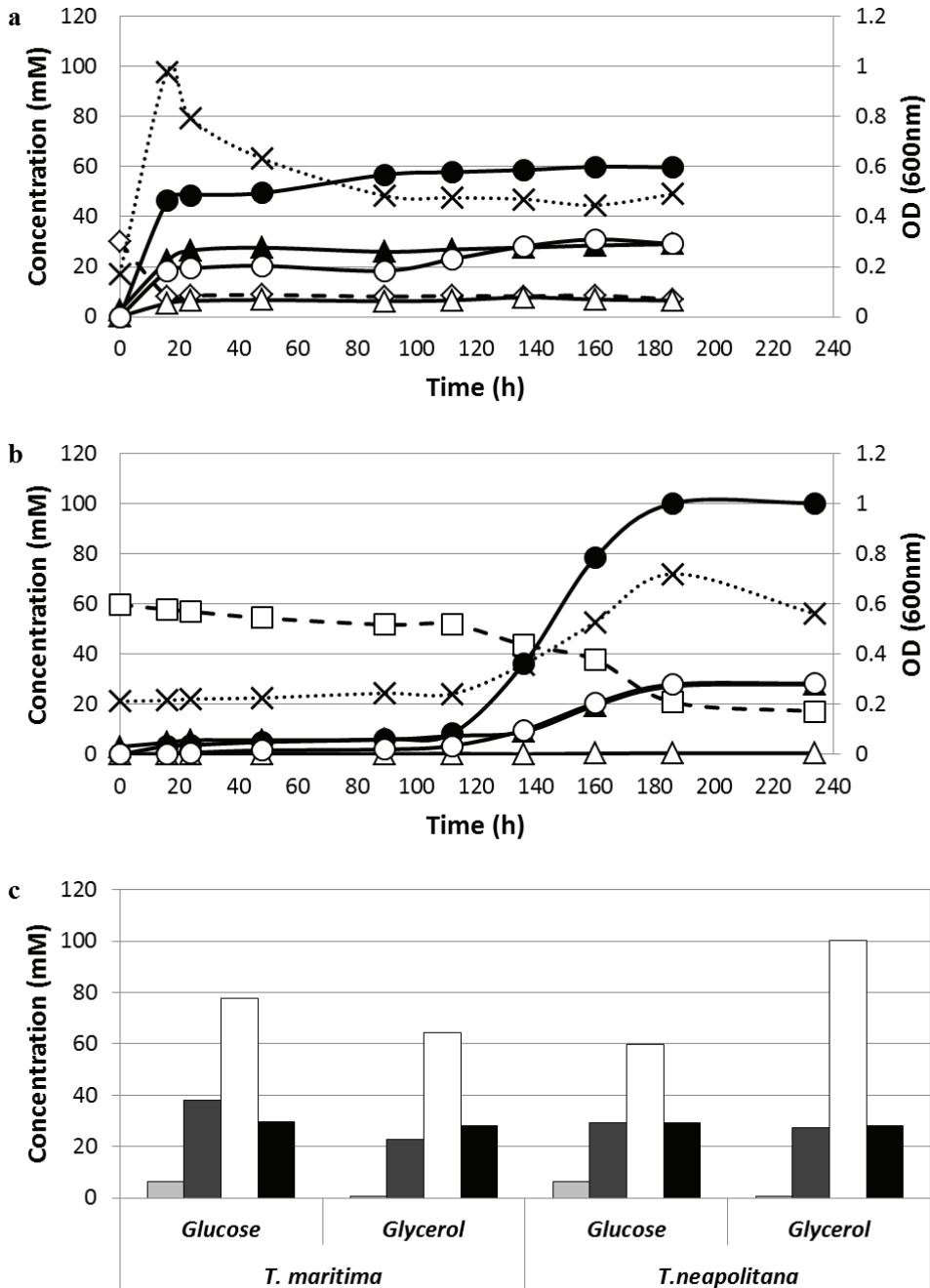


Fig. 1. (a) Growth curve of *T. neapolitana* grown on glucose (2.5 g/L); (b) Growth curve of *T. neapolitana* grown on glycerol (5 g/L). Residual glucose (dashed, \diamond), residual glycerol (dashed, \square), H₂ (\circ), CO₂ (\bullet), acetate (\blacktriangle) lactate (\triangle), and optical density (\times , secondary axis); (c) Fermentation product profile at the end of growth for *T. maritima* and *T. neapolitana* grown on glucose (2.5 g/L) and glycerol (5g/L). Lactate (light grey), acetate (dark grey), H₂ (white), CO₂ (black).

3.2. Effect of Carbon Source for *T. neapolitana* and *T. maritima*

Growth of *T. neapolitana* and *T. maritima* on glycerol (5 g/L) was compared to growth on glucose (2.5 g/L). Glucose was used as a positive control, as it is a known fermentable substrate for H₂ production by these strains with a maximum yield of up to 4 mol H₂ per mol glucose consumed [1,18]. Moreover, glucose as well as glycerol is converted partly via the Embden-Meyerhof pathway, presumably leading to identical fermentation products [13, 25].

The growth curves for *T. neapolitana* on glucose and glycerol are presented in figure 1 (a) and (b) respectively. Figure 1 (c) shows the end product formation for *T. neapolitana* and *T. maritima* using both carbon sources. Thus, *Thermotoga neapolitana* and *T. maritima* can use both carbon sources for growth and H₂ production. These results agree with earlier findings by Nelson et al. [19], Ooteghem et al and [20] Ngo et al [21]. As reported previously, *T. neapolitana* shows immediate and rapid growth on glucose (Fig. 1. (a)). Growth on glycerol, however, (Fig. 1. (b)) shows a lag phase of approximately 100 h before growth starts. Apparently, *T. neapolitana* has to adapt its metabolism to the new substrate. Repeated sub-culturing on glycerol indeed showed that the lag phase was minimized and growth commenced upon inoculation (data not shown). The growth rate on glycerol is also significantly lower than the growth rate on glucose. This is in agreement with the expected lower ATP yield during growth on glycerol compared to glucose. While growth on glucose yields both acetate and lactate, glycerol only leads to acetate (next to H₂ and CO₂).

It was observed that during growth on glucose the pH decreased from 6.8 to 5.2. The decrease of pH was attributed to the accumulation of organic acids such as acetate and lactate. High amounts of lactic acid were produced from glucose by both strains of *T. maritima* and *T. neapolitana* (Fig. 1. (c)) compared to glycerol. Acetic acid constituted more than 80% and 90% of total end products for glucose and glycerol respectively. In general, production of acetic acid favors the production of H₂ and theoretically, 3 mol of H₂ can be produced from 1 mol of glycerol in the acetate type of fermentation. The higher cumulative H₂ from glycerol is attributed to the non-branched pathway to acetate. As a result the yield for *T. maritima* using 2.5 g/L of glycerol was 2.75 mol H₂/mol glycerol (data not shown).

3.3. Effect of pH

H₂ production by *T. neapolitana* is dependent on the fermentation conditions such as pH (Fig. 2. (a)). Higher growth rates were observed at pH 6.5-7.5 while no or poor growth was detected below pH 5 and above pH 8. At pH 7 the maximum optical density (OD₆₀₀ of 0.75) and H₂ production was reached and a H₂ yield of 2.65 mol H₂/mol glycerol was observed. Therefore, the results obtained showed that a pH value between 6.5- 7.5 is optimal for the growth and H₂ production of *T. neapolitana*. Similar yields were observed by Ngo et al. [21] for *T. neapolitana* grown on glycerol in small batch cultivation system.

3.4. Effect of yeast extract

Different concentration of yeast extract (0.5, 1, 2 and 4 g/L) were used to test their influence on the fermentation performance of *T. neapolitana* grown on glycerol (Fig. 2. (b)). YE was required for growth on glycerol. Lower concentration of YE resulted in lower H₂ production (decreased glycerol consumption). Although increased YE concentration resulted in higher H₂ production levels, 4 g/L did not result in enhanced H₂ production (2.66 mol H₂/mol glycerol) with respect to 2g/L (2.65 mol H₂/mol glycerol). For *T. maritima* grown on glucose a YE concentration of 0.5 g/L resulted in maximal cell

densities [18]. Yet, when grown on glycerol, a YE concentration of 2 g/L is preferred for maximal cell densities and optimal H₂ production.

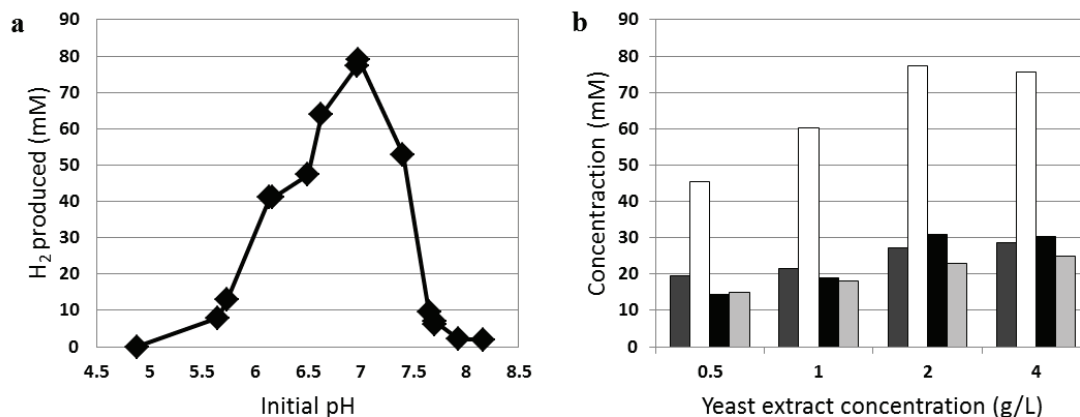


Fig. 2. (a) Effect of the initial pH on the H₂ production by *T. neapolitana* grown on glycerol. The amount of produced H₂ was determined after 168 h batch growth of *T. neapolitana* on glycerol (2.5 g/L, 27.15 mM). Initial pH indicates the pH of the media directly after inoculation (0 h); (b) Effect of yeast extract concentration on the fermentation performance for *T. neapolitana* grown on glycerol. Concentrations were determined after 148 h batch growth of *T. neapolitana* on glycerol (2.5 g/L, 27.15 mM). Glycerol consumed (dark grey), H₂ produced (white), CO₂ produced (black), acetate produced (light grey).

4. Conclusions

Here we showed that glycerol can be used by *T. neapolitana* but also by *T. maritima* for H₂ production. Thermotoga species are preferred compared to mesophilic species like e.g. *Enterobacter* species for high yield H₂ production, as no alcohols like ethanol or 1,3-propanediol are produced. H₂ yields close to the theoretical max. (3 H₂/glycerol) can be achieved using by *T. neapolitana* and *T. maritima*. The product pattern on glucose and glycerol showed that glucose is converted using a mixed-acid fermentation, whereas on glycerol a homoacetate fermentation takes place. For *T. neapolitana* optimal H₂ production was reached with a yeast concentration of 2 g/L and an initial pH of 7.

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