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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 H_2/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 H_2 production, however increasing the concentration from 2 to 4 g/L did not affect H_2 production.

Keywords: *Thermotoga*; *T. maritima*; *T. neapolitana*; hydrogen; glycerol; dark fermentation

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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 hyperthermophilc 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 H_2 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 80ºC 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 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 $NiCl_2{\cdot}6H_2O$; 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 [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 N_2 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 (OD_{600}). 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^{o}C with a eluent of 3mM H_2SO_4 (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 H_2 production of 41, 58, 65 mmol/L was observed for media A1, A2 and M3, respectively. Based on the enhanced H_2 production M3 was selected as the preferred medium, and was used in further experiments.

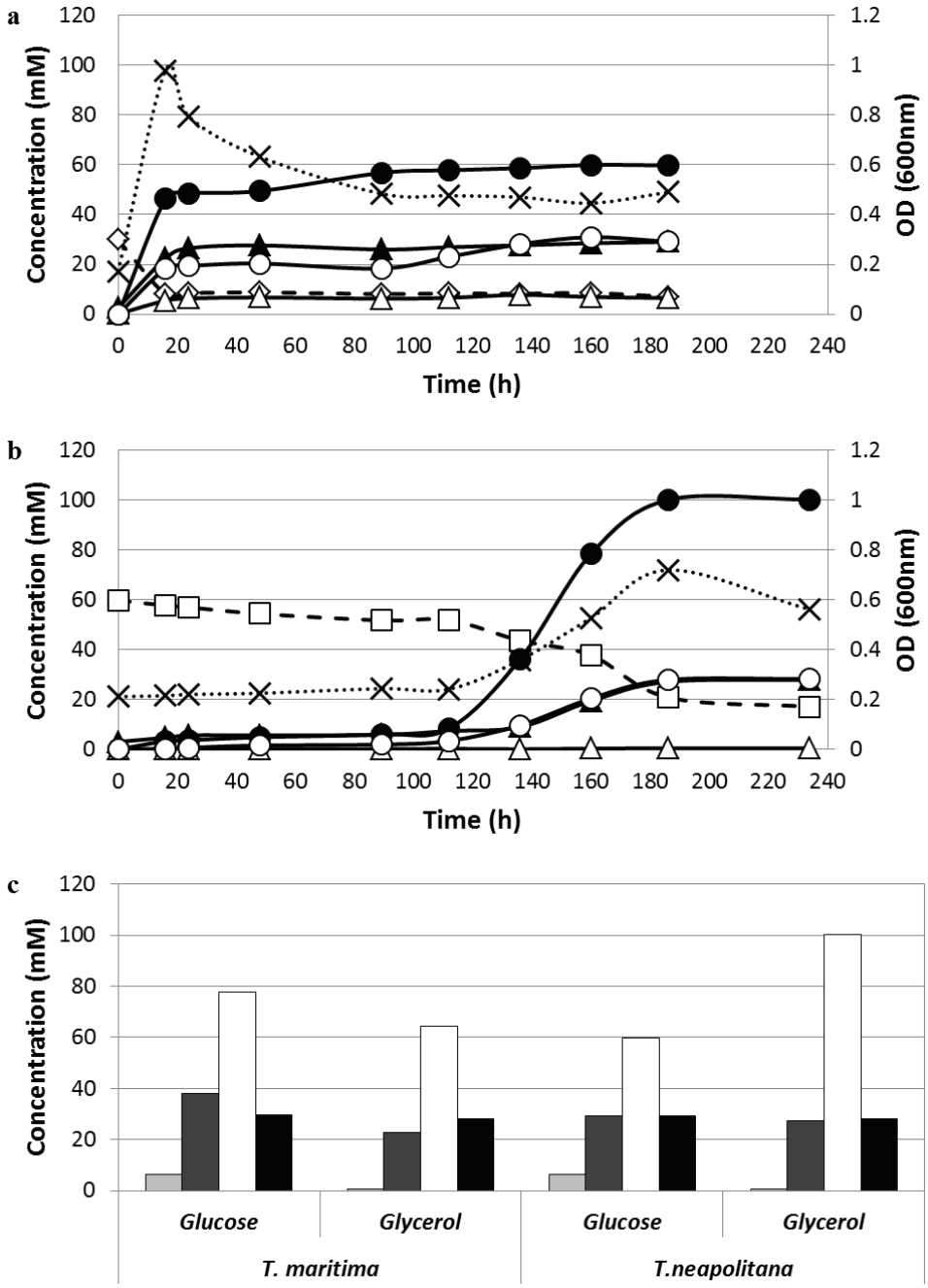

Fig. 1. (**a**) Growth curve of *T. neapolitana* grown on glucose (2.5 g/L); (**b**) Growth curve of *T. neapolitana* grown on glycerol (5 g/L). Residual glucose (dashed, ◊), residual glycerol (dashed, □), H_2 (○), CO_2 (●), acetate (▲) lactate (Δ), and optical density (×, secondary axis); (**c**) Fermentation 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), H_2 (white), CO_2 (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 H_2 production by these strains with a maximum yield of up to 4 mol H_2 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 H_2 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 H_2 and CO_2).

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 H_2 and theoretically, 3 mol of H_2 can be produced from 1 mol of glycerol in the acetate type of fermentation. The higher cumulative H_2 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 H_2/mol glycerol (data not shown).

3.3. Effect of pH

H_2 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 (OD_{600} of 0.75) and H_2 production was reached and a H_2 yield of 2.65 mol H_2/mol glycerol was observed. Therefore, the results obtained showed that a pH value between 6.5- 7.5 is optimal for the growth and H_2 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 H_2 production (decreased glycerol consumption). Although increased YE concentration resulted in higher H_2 production levels, 4 g/L did not result in enhanced H_2 production (2.66 mol H_2/mol glycerol) with respect to 2g/L (2.65 mol H_2/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 H_2 production.

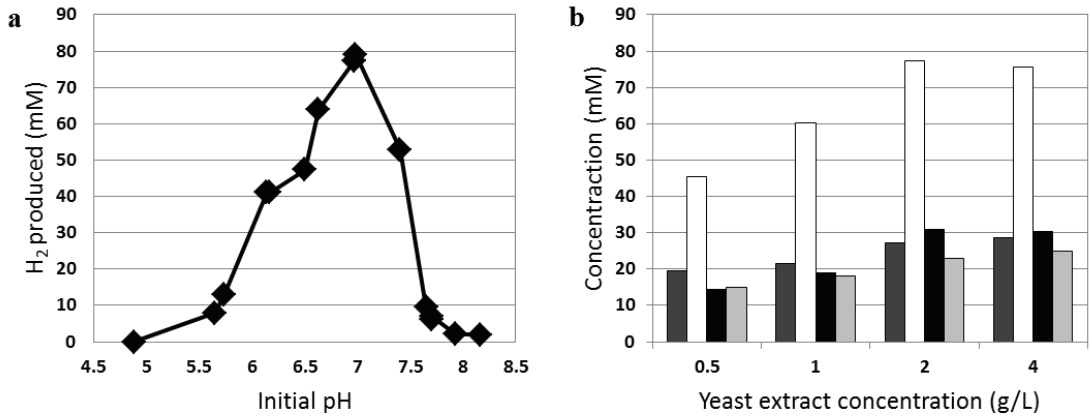

Fig. 2. (**a**) Effect of the initial pH on the H_2 production by *T. neapolitana* grown on glycerol. The amount of produced H_2 was determined after 168 h batch growth of *T. neapolitana* on glycerol (2.5 g/L, 27.15 mM). Initial pH indicates the pH of the media directly after inoculation (0 h); (**b**) 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), H_2 produced (white), CO_2 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 H_2 production. Thermotoga species are preferred compared to mesophilic species like e.g. *Enterobacter* species for high yield H_2 production, as no alcohols like ethanol or 1,3-propanediol are produced. H_2 yields close to the theoretical max. (3 H_2/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 H_2 production was reached with a yeast concentration of 2 g/L and an initial pH of 7.

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References

[1] Van Ooteghem SA, Beer SK, Yue PC. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. *Appl Biochem Biotechnol* 2002;**98**:177–89

[2] Kadar Z, De Vrije T, Van Noorden G, Budde M, Szengyel Z, Reczey K, et al. Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. *Appl Biochem Biotechnol* 2004;**114**:497–08.

[3] van Niel EW, Claassen PA, Stams AJ. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. *Biotechnol Bioeng* 2003;**81**:255–62.

[4] Tong Z, Hong L, Herbert HPF. Biohydrogen production from starch in wastewater under thermophilic condition. *J Environ Manage* 2003;**69**:149-56

[5] Kim SH, Han SK, Shin HS. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *Int J Hydrogen Energy* 2004;**29**:1607–16.

[6] Kapdan IK, Kargi F. Hydrogen production from waste materials. *Enzyme Microb Technol* 2006;**38**:569–82.

[7] Nguyen DTA, Han SJ, Kim JP, Kim MS, Oh YK, Sim SJ. Hydrogen production by the hyperthermophilic eubacterium, *Thermotoga neapolitana*, using cellulose pretreated by ionic liquid. *Int J Hydrogen Energy* 2008;**33**:5161–8

[8] Kim MS, Lee DY. Fermentative hydrogen production from tofu-processing waste and anaerobic digester sludge using microbial consortium. *Bioresour Technol* 2010;**101**:548–52.

[9] Yazdani SS, Gonzalez R. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Curr Opin Biotechnol* 2007;**18**:213–19.

[10] Katryniok B, Paul S, Capron M, Dumeignil F.. Towards the sustainable production of acrolein by glycerol dehydration. *ChemSusChem* 2009;**2**:719-30.

[11] Rainey FA, Donnison AM, Janssen PH, Saul D, Rodrigo A, Bergquist PL et al. Description of *Caldicellulosiruptor saccharolyticus* gen., nov., sp.nov: an obligately anaerobic, extremely thermophilic, cellulolytic bacterium. *FEMS Microbiology Letters,* 1994;**120**:263-66.

[12] Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N. Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. *J Biosci Bioeng* 2005;**100**:260–65.

[13] Temudo MF, Poldermans R, Kleerebezem R. Van Loosdrecht MCM. Glycerol fermentation by (open) mixed cultures: a chemostat study. *Biotechnol Bioeng* 2008;**100**:1088–98.

[14] Biebl H, Zeng AP, Menzel K,Deckwer WD. Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by *Klebsiella pneumoniae*. *Appl Microbiol Biotechnol* 1998;**50**:24–29.

[15] Mathews J, Wang G. Metabolic pathway engineering for enhanced biohydrogen production. *Int J Hydrogen Energy* 2009;**34**:7404–16.

[16] Verhaart MRA, Bielen AAM, Oost Jvd, Stams AJM, and Kengen SWM.. Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal. *Environ Technol* 2010;**31**:993 – 1003.

[17] Wiegel J, Ljungdahl LG, Demain AL. The importance of thermophilic bacteria in biotechnology. *Crit Rev Biotechnol* 1985;**3**:39–108

[18] Schroder C, Selig M, Schonheit P. Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima* involvement of the Embden-Meyerhof pathway. *Arch Microbiol* 1994;**161**:460–70.

[19] Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH et al. Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. *Nature* 1999;**399**:323–29.

[20] Van Ooteghem SA, Jones A, Van Der Lelie D, Dong B, Mahajan D. H_2 production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions. *Biotechnol Lett* 2004;**26**:1223–32.

[21] Ngo TA, Kim MS, Sim SJ. High–yield biohydrogen production from biodiesel manufacturing waste by *Thermotoga neapolitana. Int J Hydrogen Energy* 2011;**36**:5836–42.

[22] Eriksen NT, Riis ML, Kynd NH, Iversen N. H_2 synthesis from pentoses and biomass in *Thermotoga* spp.. *Biotechnol Lett* 2011;**33**:293–00

[23] Damsté JSS, Rijpstra WIC, Hopmans EC, Schouten S, Balk M, Stam A JM. Structural characterization of diabolic acid–based tetraester, tetraether and mixed ether/ester, membrane–spanning lipids of bacteria from the order *Thermotogales*. *Arch Microbiol* 2007;**188**:629–41.

[24] Kongjan P, Min B, Angelidaki I. Biohydrogen production from xylose at extreme thermophilic temperatures (70°C) by mixed culture fermentation. *Water Res* 2009;**43**:1414–24.

[25] Selembo PA, Perez JM, Lloyd WA, Logan BE. Enhanced hydrogen and 1,3–propanediol production from glycerol by fermentation using mixed cultures. *Biotechnol Bioeng* 2009;**104**:1098–106.