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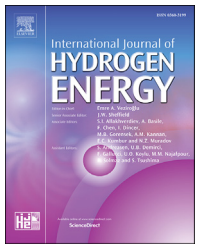

Thermophilic versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: Biohydrogen production and active microbial community

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ABSTRACT

Dark fermentative biohydrogen production in a thermophilic, xylose-fed (50 mM) fluidised bed reactor (FBR) was evaluated in the temperature range 55–70 °C with 5-degree increments and compared with a mesophilic FBR operated constantly at 37 °C. A significantly higher ($p = 0.05$) H_2 yield was obtained in the thermophilic FBR, which stabilised at about 1.2 mol H_2 mol^{-1} xylose (36% of the theoretical maximum) at 55 and 70 °C, and at 0.8 mol H_2 mol^{-1} xylose at 60 and 65 °C, compared to the mesophilic FBR (0.5 mol H_2 mol^{-1} xylose). High-throughput sequencing of the reverse-transcribed 16S rRNA, done for the first time on biohydrogen producing reactors, indicated that *Thermoanaerobacterium* was the prevalent active microorganism in the thermophilic FBR, regardless of the operating temperature. The active microbial community in the mesophilic FBR was mainly composed of *Clostridium* and *Ruminiclostridium* at 37 °C. Thermophilic dark fermentation was shown to be suitable for treatment of high temperature, xylose-containing wastewaters, as it resulted in a higher energy output compared to the mesophilic counterpart.

Introduction

H_2 is a carbon free fuel considered as a promising candidate to replace fossil fuels in the near future [1]. Although hydrocarbons are currently the main feedstock for H_2 production, biomass is a renewable and environmentally friendly alternative feedstock [2]. Dark fermentation is the most studied among the biological H_2 production technologies because the variety of usable organic substrates and the high achievable conversion rates may promote the scale-up of the process [3]. However, due to the thermodynamics of the reactions involved, which are more favourable at high temperature and low H_2 partial pressure, operation and optimisation of full-scale dark fermentation is more challenging than traditional anaerobic digestion [4].

Many pathways are possible for dark fermentation, depending on the microbial species, operating parameters,

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and the substrate used. Glycolysis is the most common route for degradation of hexoses by *Clostridium* [5] and most thermophiles, including *Thermoanaerobacter* [6]. Glucose is oxidized to pyruvate, resulting in the generation of reduced nicotinamide adenine dinucleotide (NADH) and energy in the form of adenosine triphosphate [7]. Pyruvate may be further oxidized to acetylcoenzyme A through reduction of ferredoxin and then to volatile fatty acids (VFAs), or alcohols [7]. Metalloenzymes called hydrogenases use NADH or reduced ferredoxin as electron donor for proton oxidation [8], resulting in the formation of molecular H_2. The oxidation of glucose to H_2 and CO_2 yields 12 mol H_2 mol^{-1} glucose. However, the dehydrogenation of acetate to CO_2 is endergonic, and the spontaneous oxidative process will thus end with acetate production, yielding only 4 mol H_2 mol^{-1} glucose [9]. This thermodynamic limitation is also valid for pentose sugars such as xylose, which will then yield a maximum of 3.33 mol H_2 mol^{-1} xylose.

In practice, the H_2 yield by mixed cultures varies from 14% to 70% of the theoretical limit [10]. For high H_2 partial pressures (>60 Pa), proton reduction by NADH is not thermodynamically favourable and the reaction switches, e.g. to the butyrate pathway [11], resulting in a lower H_2 yield. This can be mitigated by operating dark fermentation in well mixed systems, such as fluidised bed reactors (FBRs), in which the turbulent mixing regime favours the stripping of the produced H_2 [12]. Temperature and pH also strongly affect the microbial community and thus, the substrate degradation pathway [13]. Butyrate accumulation can trigger solventogenesis [14], which does not yield H_2. Some microorganisms, including various *Clostridium* sp., are facultative autotrophic and can reduce CO_2 with H_2 forming acetate [10]. Other known H_2-consuming microorganisms include hydrogenotrophic methanogens, propionate producers, and sulphate or nitrate reducing microorganisms [15]. Enhancing the growth of H_2 producers while avoiding the growth of H_2 consuming microorganisms in dark fermentative bioreactors is still an open challenge [16].

H_2 production at high temperatures can be advantageous in terms of H_2 yield and production rates [17,18]. High temperature positively affects the kinetics of the oxidative reactions and the growth of microorganisms [19]. Furthermore, the direct conversion of sugars to acetate becomes thermodynamically more favourable as the temperature increases, thus resulting in a high H_2 yield [20]. Thermophilic anaerobic microorganisms such as *Thermotoga* and *Thermoanaerobacterium* are excellent H_2 producers, as they use most of the reductants produced during glycolysis to form H_2, allowing yields between 3 and 4 mol H_2 mol^{-1} hexose [20]. Although H_2 yields from pure cultures are typically higher [5], mixed cultures are preferable for industrial application, as they offer more stability and versatility, and sterilisation is not required [13]. Most studies on H_2 production at high temperature by mixed cultures have been conducted at 55, 60, or 70 °C, generally obtaining higher H_2 yields than in mesophilic trials with a similar inoculum and substrate [18,21,22]. Sources of inoculum used for thermophilic dark fermentation include e.g. sewage sludge [18,23], anaerobic sludge or digestates [21,24–28], animal dung or slurry [29,30], hot spring sediment [31,32], and biomass from previous laboratory-scale H_2 production experiments [33–39].

Handling and processing of organic substrates and the low H_2 yield are two of the main deterrents for the establishment of dark fermentation at commercial scale [40]. Despite the higher H_2 yield obtained at high temperatures, the net energy gain (the difference between the energy input needed to heat the reactor and output) seems to be indirectly proportional to the operation temperature [41]. However, some industrial wastewaters, such as thermomechanical pulping wastewaters, are produced at high (50–70 °C) temperatures [42] and could be treated on site avoiding cooling and minimising energy loss. Such wastewaters contain readily fermentable sugars, both hexoses (e.g. glucose) and pentoses (e.g. xylose), suitable for H_2 production by dark fermentation. Continuous dark fermentation of glucose has been widely studied at various temperatures [35,37,39,43,44] while much less attention was given to dark fermentation of xylose, especially at high temperature. In a previous study, H_2 production from xylose was compared in batch cultures at 37, 55 and 70 °C using heat treated (90 °C, 15 min) activated sludge from a wastewater treatment plant as the inoculum [18]. That study showed effective H_2 production at 55 °C, but not at 70 °C. However, the effect of temperature in the 55–70 °C temperature range is worth investigating, as a difference of a few degrees can affect the microbial community inside the reactor and thus, the H_2 production efficiency [44].

Recently, the establishment of next-generation sequencing techniques has improved the knowledge on H_2-producing microbial communities. Etchebehere et al. [45] performed 454 pyrosequencing on microbiological samples from 20 H_2 producing lab-scale bioreactors operated within a temperature range of 25–37 °C. Although the microbial communities were diverse due to the different operating conditions of the bioreactors, the authors observed a predominance of *Firmicutes* and distinguished high-yield H_2 producers (*Clostridium*, *Kosmotoga*, and *Enterobacter*), low-yield H_2 producers (*Veillonellaceae*) and competitors (*Lactobacillus*). Nitipan et al. [46] reported *Thermoanaerobacterium* as the dominant genus in a thermophilic (60 °C) sequencing batch reactor producing H_2 from palm oil mill effluent. Zhang et al. [47] showed that *Firmicutes* such as *Caldanaerobius*, *Caldicellulosiruptor* and *Thermoanaerobacter* became dominant in a hyperthermophilic (70 °C) H_2 producing, glucose-fed chemostat. To date, analysis of dark fermentative microbial communities by next-generation sequencing has been mainly based on the presence of 16S rRNA genes, which provides information on the structure of the microbial community. However, an analysis based on the expression of 16S rRNA genes describes more accurately the composition of the microbial community actively involved in dark fermentation [48].

This study aims to evaluate, for the first time, how dark fermentative H_2 production and the composition of the active microbial community are affected by a stepwise (5 °C) temperature increase in the 55–70 °C temperature range in a xylose-fed FBR inoculated with heat treated activated sludge. A second FBR was operated in parallel with the same inoculum, but at a lower temperature (37 °C) in order to compare its performance to the thermophilic counterpart, prior to increasing the temperature to 55 °C to observe the response of this mesophilic microbial community to the temperature shift.