Applied Energy 198 (2017) 77–87

Contents lists available at ScienceDirect

Applied Energy

journal homepage: www.elsevier.com/locate/apenergy

# Dark-fermentative biohydrogen pathways and microbial networks in continuous stirred tank reactors: Novel insights on their control

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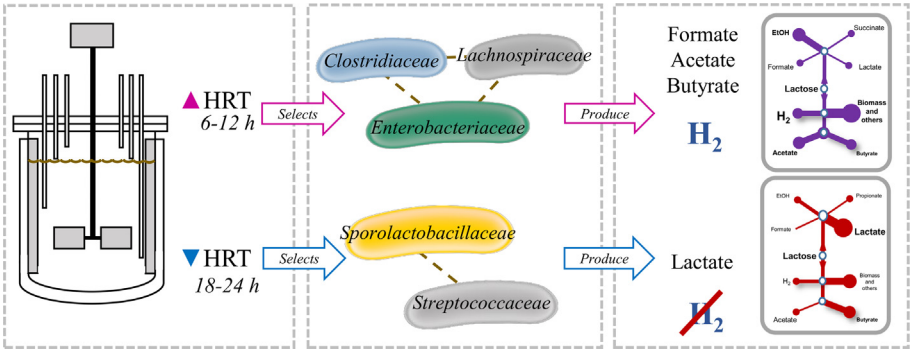
## HIGHLIGHTS

- Dark fermentation microbial community was strongly shaped by HRT.
- A maximum volumetric hydrogen production rate of 2000 mL/L-d was found at 6 h of HRT.
- Two different microbial communities and their interactions were identified.
- Short HRT (6–12 h) enriched *Clostridiaceae-Lachnospiraceae-Enterobacteriaceae*.
- Large HRT (18–24 h) negatively affected the performance of dark fermentation.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 9 February 2017
Received in revised form 29 March 2017
Accepted 15 April 2017

*Keywords:*
Biohydrogen
Dark fermentation
Hydrogen-producing bacteria
Metabolic network
Lactic acid bacteria

## ABSTRACT

In the present work, the influence of hydraulic retention time (HRT) on dark fermentation metabolism was evaluated through the operation and analysis of a series of four continuous stirred tank reactors (CSTR) at four HRT ranging from 6 h to 24 h. A maximum volumetric hydrogen production rate (VHPR) of 2000 ± 149 mL/L-d corresponding to an $H_2$ yield of 0.86 $mol_{H2}/mol_{lactose}$ was observed at 6 h HRT. In depth analysis of metabolite profiles and microbial communities showed that low values of HRT favored the emergence of a community dominated by *Clostridiaceae-Lachnospiraceae-Enterobacteriaceae*, which performed metabolic pathways co-producing hydrogen. In contrast, long HRT led to the establishment of *Sporolactobacillaceae-Streptococcaceae* microbial community that outcompeted hydrogen producing bacteria and was responsible of lactate production. Results suggested that these two communities mutually excluded themselves and HRT can act as an operational parameter to control the microbial communities and consequently the related metabolic pathways.



## 1. Introduction

Biohydrogen is worldwide considered as one of the most promising alternatives to substitute fossil fuels in a near future. Indeed, hydrogen is not only characterized by its high density of energy (123 kJ/g ~ 2.75), but also the efficiency of its conversion to electric energy is relatively high, and its utilization does not generate any greenhouse gases. Amongst the technologies available to produce hydrogen, biological processes are environment friendly and can convert a wide variety of abundant organic biomass at low cost. In particular, biological production of hydrogen by dark fermentation (DF), so called biohydrogen, can be emphasized for its large use of sustainable substrates, the high hydrogen

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http://dx.doi.org/10.1016/j.apenergy.2017.04.051
0306-2619/

production rates, and its simplicity of operation [1]. In contrast with the photo-fermentation processes, DF does not require light to occur, thus reactor design is simpler and its operation is not limited by light-darkness natural cycles.

In brief, a fermentation process is the biological oxidation of organic compounds where the same substrate molecule plays a role as a carbon source, an electron donor- and an electron-acceptor, i.e. a part of the molecule is oxidized while another part is reduced [2]. In particular, the DF process can be defined as the partial oxidation of organic substrates (mainly carbohydrates) without external electron acceptor. DF leads to the production of low weight organic molecules (volatile fatty acids – VFA – and alcohols) altogether with hydrogen generation. Such metabolic process can be carried out by mixed cultures of bacteria, the most representative members being related to *Prevotella*, *Lactobacillus*, *Clostridium*, *Selenomonas*, *Megasphaera* and *Enterobacter* genera [3].

Metabolically, the maximum theoretical hydrogen yield of DF is 4 mol of hydrogen per mol of glucose consumed through the acetate pathway [4]. In practice, $H_2$ yields reported for mesophilic cultures [5–8] are about 1.3 $mol_{H2}/mol_{glucose}$ in average, while only few studies have reported hydrogen yields beyond 3 $mol_{H2}/mol_{hexose}$ [6]. Low hydrogen yields are probably linked to the complexity of microbial communities and metabolic pathways presented in the DF.

In a dark fermentative community some members such as *Clostridium* and *Enterobacter* genera (hydrogen producing bacteria, HPB) are efficient hydrogen producers while others play different roles not necessarily linked to hydrogen production, for instance, homoacetogens and lactic acid bacteria (LAB). On one hand, homoacetogens can use carbon dioxide or carbon monoxide and hydrogen as sole carbon and energy sources under anaerobic conditions, along with the synthesis of acetate [9–12]. Previous studies showed homoacetogenesis as the cause of 36–56% of the total acetate observed in the fermentation media, resulting in hydrogen productivities 45–90% lower than expected [10]. Other authors reported that hydrogen consumption by homoacetogens was equivalent to 250 mmol/d at 8 h of HRT in UASB reactors [13]. To avoid this type of metabolism, several strategies consisting in the reduction of hydrogen accumulation have been suggested (e.g. gas sparkling [14]). On the other hand, LAB constitutes a microbial group commonly found in DF systems (e.g. [15–19]) which includes microorganisms of the families *Lactobacillaceae*, *Enterococcaceae*, *Streptococcaceae*, *Sporolactobacillus*, etc. Despite of their ubiquity, the role of LAB in DF has been scarcely studied [20]. Some authors argued that LAB compete with hydrogen producers for carbon sources [21], while others have widely discussed that the excretion of bacteriocins could be the main cause of DF failure [18,19].

Up to date, most of the reports in literature about LAB and other important microbial groups have been studied circumstantially. In consequence, there is still knowledge scarcity about their effects on fermentation performance, metabolic pathways and their roles in the microbial community of DF. A full understanding of these issues is fundamental to conduct better control and advance towards the implementation of the biohydrogen production technology at the industrial scale. In this direction, the study of the factors that determine the occurrence of these groups in dark fermentative systems is a pending task.

Therefore, throughout the experiments carried out in this work, hydraulic retention time (HRT) was evaluated as a potential factor to shape the metabolic pathways and microbial communities in continuous dark fermentative systems. Due to its simplicity and practicality in real life operation in comparison with other strategies, HRT could be of high importance for metabolic and microbial community control. Moreover, a microbial network analysis was used to reveal the interactions among the involved species. This methodology provides highly important information for the engineering and/or design of microbial communities, which is an alternative to enhance hydrogen production.

## 2. Materials and methods

### 2.1. Inoculum source and substrate

Disaggregated anaerobic sludge from a full-scale municipal wastewater treatment plant (Marseille, FR) was used as initial source of microorganisms. Before inoculation, the sludge was thermally treated by boiling during 2 h. Seed sludge was added into a continuous stirred tank reactor (CSTR1) of 3.1 L total volume and 2 L working volume (APPLIKON Biotechnologies, USA) at a final concentration of 4.5 g/L of volatile suspended solids (VSS). The reactor was started in batch mode for 24 h, whereupon the bioreactor was operated in continuous mode for 25 d with a HRT of 6 h. When CSTR1 reached a stable state (lactose degradation efficiency, 46 ± 2%; VHPR, 2448 ± 461 $L_{H2}$/L-d; $H_2$ yield, 0.89 $mol_{H2}/mol_{lactose}$) 10 L of effluent were recovered and stored in 4 containers (2.5 L

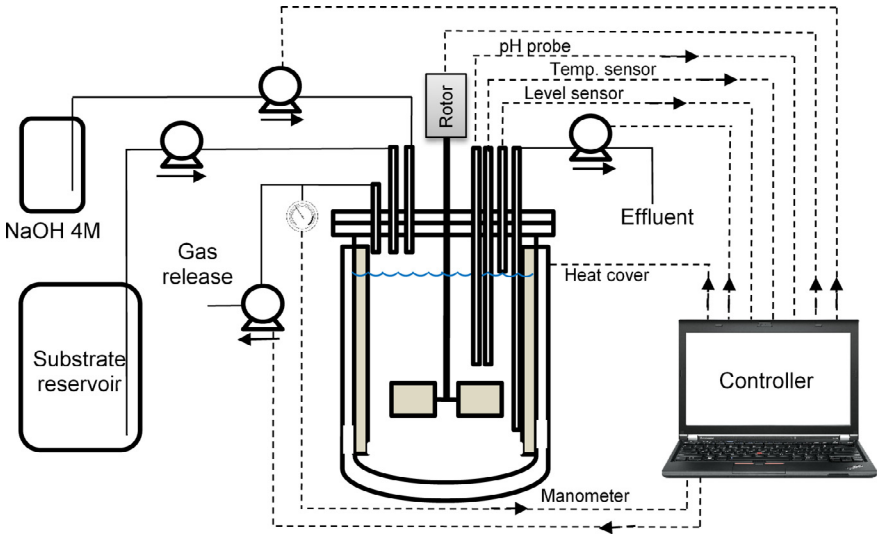


**Fig. 1.** Schematic representation of the experimental set-up used in this work.