INTERNATIONAL JOURNAL OF HYDROGEN ENERGY XXX (2014) 1–6

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Comparison of hydrogen-producing bacterial communities adapted in continuous and discontinuous reactors

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ARTICLE INFO

Article history:
Received 19 October 2013
Received in revised form
12 December 2013
Accepted 5 January 2014
Available online xxx

Keywords:
Biohydrogen
Anaerobic granules
Continuous operation
Discontinuous operation
Microbial community
DGGE

ABSTRACT

We evaluate the bacterial composition during the anaerobic granules adaptation for hydrogen production using continuous and discontinuous feeding-regime. Adaptation was induced by employing short hydraulic retention times and low pH. The microbial community analysis revealed that both discontinuous and continuous adaptation strategies resulted in the selection of different microorganisms despite the use of the same initial inoculum. The dominant microorganisms present in the continuous process belong to the genus *Escherichia* and *Clostridium*; while in the discontinuous adaptation prevailed Clostridiales. The different feeding-regimes applied not only reduced the diversity, but also the composition of the microbial community. The Jaccard's and Shannon–Wiener indexes showed that the different operational strategies applied not only reduced the diversity, but also the composition of the microbial community.

1. Introduction

The selection of hydrogen-producing bacteria communities has important repercussions during the start-up phase of any reactor [1]. The microbial community composition has a strong effect on the process efficiency [2]. Pure and mixed bacterial cultures have been applied as source of inoculum to produce hydrogen [3,4]. Different genus of microorganism including *Clostridium*, *Escherichia*, *Enterobacter*, *Bacteroides* and *Bacillus* has been reported as the hydrogen producers in biological systems [5–7].

Up to now, the anaerobic sludge has been the main inoculum source to produce hydrogen [3,4]. In the anaerobic granules different types of microorganisms are distributed along the granule forming differentiated layers [8]. Fermentative bacteria are distributed mainly at the outer layer of the granule and are in contact with the fed substrate. The acetogenic microorganisms, including hydrogen-producing bacteria, are located in the middle of the granule [8].

From a practical point of view, bacterial consortia are more suitable than pure cultures since it is possible to produce hydrogen in non-sterile conditions [9]. Although mixed cultures can be more robust to the operational changes than pure

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http://dx.doi.org/10.1016/j.ijhydene.2014.01.014

cultures, their main drawback is the presence of hydrogen consuming microorganisms [4].

Different pretreatment methods have been evaluated to produce hydrogen by inactivating those hydrogen-consuming microorganisms [10–12]. However, its use at large scale can result limitative and impractical [13]. A practical methodology to obtain hydrogen-producing-bacteria was already reported by Ref. [1]. The adaptation of anaerobic granules was obtained using the selection pressure imposed by a low pH and short hydraulic retention time (HRT).

It has been observed that the feeding-regime (continuous or discontinuous) of bioreactors influences the hydrogen productivity [1] and this can be attributed to the microbial community variation, but the comparison of the microbial community under those feeding-regimes has not been already reported. Ning et al. [14] observed the microbial community evolution during adaptation in a continuous upflow anaerobic sludge blanket (UASB). These authors found that after the mature hydrogen-producing granules were formed, the microbial community structure in reactor tends to stabilize. Bacteria diversity variation has been observed in discontinuous process. Arreola-Vargas et al. [15] observed a low bacterial diversity in the stages with high hydrogen production and vice versa. These studies suggests that low diversity and stable microbial community in a reactor could be obtained when high production of hydrogen is observed, independently of the reactor-feeding regime.

The objective of this study was to evaluate the bacterial composition during the anaerobic granules adaptation for hydrogen production using continuous and discontinuous feeding regime.

2. Materials and methods

2.1. Experimental procedure

The experiment was carried out with two reactors with different feeding regime [1]. An anaerobic sequencing batch reactor was operated under three HRT. The HRT was gradually decreased (24, 12 and 6 h). The pH was controlled (pH controller, Hanna Instruments) at 5.5 ± 0.1 by adding 2 N NaOH [16]. The second reactor was a continuous UASB reactor operated during 66 days. The pH was controlled (pH controller, Hanna Instruments) at 4.5 ± 0.1 and the HRT was fixed to 5.5 h. For both cases, the Organic Loading rate (OLR) was maintained at 5 g-glucose L^{-1} d^{-1}. The reactors were operated at mesophilic conditions (35 ± 1 °C) by using a ribbon heater with a temperature controller (Thermo Scientific).

For this study, adaptation of granular sludge considered the hydrogen production and the suppression of the methane. Adaptation was conducted according to [1]. The reactor was considered adapted when the hydrogen content in the gas was stable (variations were lower than 10%).

2.2. Analytical assessment

2.2.1. Standard measurements of wastewater parameters

Biogas production was measured by collecting it into an inverted glass cylinder filled with water. The biogas composition (H_2, CH_4, and CO_2), volatile fatty acids (acetic, butyric, propionic, and valeric acids) and ethanol were chromatographically analyzed according to [1]. The glucose concentration was measured by the phenol-sulphuric acid method described by Dubois et al. [17] with glucose as standard.

2.2.2. Microbial community analysis

Molecular techniques were applied to analyze the microbial community of the reactors, including Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE) techniques. Total DNA was extracted and purified from the granular samples using MoBio Ultraclean Soil DNA Kit according to the manufacturer's instruction. The DNA extraction was verified by means of an agarose gel at 1%. The PCR mixture (30 μl total volume) contained 12.5 μL of TopTaq PCR Master Mix Qiagen (0.25 units of DNA Polymerase, 200 μM of each dNTPs, 1.5 mM $MgCl_2$ PCR buffer), 5 μL of template DNA, 10 mM of each primer, 4 μL of $MgCl_2$ (50 mM) and 6.5 μL of RNases- free water.

Two universal bacteria primers sets were used. 534R (ATT ACC GCG GCT GCT GG) and 46F-GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GCC TAA CAC ATG CAA GTC). The following conditions were used for the PCR amplification: 2 min at 94 °C for initial denaturalization and 30 cycles (1 min at 94 °C, 1 min at 54 °C for alignment and 1 min at 72 °C for elongation), and 7 min at 72 °C for final extension step. PCR reactions were performed in a Gradient Palm-Cycler (Corbett research). The PCR amplification products were confirmed by electrophoresis with a 1% (w/v) agarose gel.

DGGE was performed using a DCode Universal Mutation Detection System (BioRad Laboratories) as described by Muyzer et al. [18]. The 8% (w/v) acrylamide solution was used to perform a gel with denaturant gradients ranging from 40% to 60% for universal-bacteria DNA fragment. Electrophoresis was conducted in a 0.5× TAE (40 mM Tris, 40 mM acetic acid, and 10 mM EDTA $2Na{\cdot}2H_2O$) buffer solution at 80 V and 60 °C for 17 h.

The gels were stained for 40 min with SYBR Green (Invitrogen), destained with deionized water for 10 min, visualized UV radiation and photographed using Gel Logic 200 Imaging System (Eastman Kodak Company). DGGE bands selected for sequence analysis were manually excised from the gel and placed in 80 μl of RNase-free water (Sigma–Aldrich). DNA was eluted through a freezing-and-thawing procedure: samples were stored at −20 °C for 16 h, then at room temperature for 3 h and finally thawing overnight at 4 °C. One microliter of each aliquot was used as a template for PCR. The eluted DNA fragments were re-amplified with primer sets 46F (GCCTAACACATGCAAGTC)/534R. The amplification was confirmed by electrophoresis on a 1% (w/v) agarose gel, obtaining partial 16s sequence in PCR products.

The identification of the obtained sequences was performed by the use of the Basic Local Alignment Search Tool (BLAST) software [19], taking into account the results that may contain a gap. Relative microbial abundances were estimated on bacterial DGGE gel using band intensities by a gel electrophoresis image analysis software GelAnalyzer 2010a (GelAnalyzer.com). DGGE was analyzed, taking into account the relative microbial abundances, by PAST software [20]. The

Please cite this article in press as: Hernández-Mendoza CE, et al., Comparison of hydrogen-producing bacterial communities adapted in continuous and discontinuous reactors, International Journal of Hydrogen Energy (2014), http://dx.doi.org/10.1016/j.ijhydene.2014.01.014