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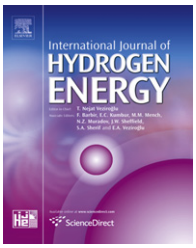

Microbial diversity analysis of long term operated biofilm configured anaerobic reactor producing biohydrogen from wastewater under diverse conditions

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ABSTRACT

This communication provides an insight into the composition of the microbial community survived in the biofilm configured anaerobic reactor operated for biohydrogen (H_2) production using wastewater as substrate under diverse conditions for past four years. PCR amplified 16S rDNA product (at variable V3 region using universal primers 341F and 517R) was separated by using denaturing gradient gel electrophoresis (DGGE) to identify the diversity in microbial population survived. The phyologenetic profile of the bioreactor showed significant diversity in the microbial community where major nucleotide sequences were affiliated to Class Clostridia followed by Bacteroidetes, Deltaproteobacteria and Flavobacteria. *Clostridium* were found to be dominant in the microbial community observed. The controlled growth conditions, application of pre-treatment to biocatalyst, operation with specific pH and variation in substrate composition are reasoned for the robust acidogenic culture identified in the bioreactor. Most of the operational taxonomic units (OTUs) observed in the bioreactor are capable to undergo acetate producing pathway, feasible for effective H_2 production.

1. Introduction

The rapid growth and industrialization of nations and burgeoning economies have resulted in a steep increase in fossil fuel production and utilization. This has not only put a severe strain on the already depleting fossil fuels but also resulted in an alarming increase in pollution levels across the globe. Thus, the rapid development of alternative, renewable, carbon-neutral and eco-friendly fuels is of paramount importance and biofuels perfectly fit this bill. In this realm, biohydrogen (H_2) production is considered as one of the opportunistic and sustainable way to meet the future energy demand. One of the possible biological approaches for producing biohydrogen is to convert negative valued organic waste under anaerobic fermentation into valuable sources [1,2]. Much of the research on biohydrogen so far was concentrated on the optimization studies, while, relatively less stress appears on the community structure analysis of the biocatalyst (consortia) used [3,4]. Nature of the fermentation products depends on the microbial diversity present in the bioreactor [5]. Microbial community/diversity greatly influences the production of volatile fatty acids (VFA) which is one of the crucial factor for H_2 production [6,7]. Generally, microbial diversity gets influenced by the operating conditions used viz., nature of substrate, substrate loading rate, hydraulic retention time (HRT), operating pH, etc. Correlating the operation data with the microbial community profile will help to understand the process happening in the bioreactor and

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further facilitate to optimize the system conditions for effective output.

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) is generally used to determine the existing and dominant members of the microbial community. The strength of DGGE as a screening method for diversity lies in its ability to monitor the community structure in response to the changes in experimental parameters by overcoming some of the limitations in cultural techniques [8]. Full-cycle 16S rDNA analysis has allowed microbiologists to describe the diversity of individuals within the population and to identify novel organisms [9]. The 16S ribosomal DNA genes are useful for such studies as these genes are present in all bacteria and comparison of sequences of 16S ribosomal DNA genes has been well established as a standard method for the identification of bacteria [10]. The genes contain both conserved and variable regions which can be used respectively for primer designing in PCR amplification and to distinguish sequences from each other. All the 16S rDNA genes are equally accessible for the amplification, whether whole cell or extracted, physiological state of the cell doesn't effect the amplification and it is the basic principle behind the success of this technique [10]. An attempt was made in this communication to study the microbial diversity persisted in anaerobic biofilm reactor being operated for past four years to produce H_2 from different types of wastewaters under highly variable conditions (Table 1) employing PCR-DGGE based 16S rDNA molecular technique. The main focus of this study was to interpret the relation among the persistent microbial communities established during the long term operation of the bioreactor with the function of bioreactor performance.

2. Materials and methods

2.1. Anaerobic consortia

Anaerobic mixed microflora from an operating laboratory scale upflow anaerobic sludge blanket (UASB) reactor treating chemical wastewater was used as parent inoculum for the startup of the bioreactor. Prior to use, the seed inoculum was sieved to remove any stone, sand and other coarse material. Prior to inoculation, dewatered sludge was subjected to cyclic pre-treatment sequence (four times) changing between heat-shock (100 °C, 2 h) and acid [pH 3 adjusted with orthophosphoric acid (88%), 24 h] treatment to restrain the growth of methanogenic bacteria (MB) and at the same time to selectively enrich the H_2 producing acidogenic bacteria (AB) [11–13]. The resulting enriched mixed culture was used as parent inoculum for the startup of the bioreactors. During operation occasionally, the reactors were subjected to chemical pre-treatment by employing 2-bromoethane sulphonic acid sodium salt (BESA, 0.2 g/l; 24 h).

2.2. Anaerobic bioreactor

Bench scale, biofilm configured, anaerobic reactor was designed and fabricated in laboratory using 'perspex' material to operate in upflow mode with a pre-defined volume of 1.8 l and a gas holding capacity of 0.3 l (L/D ratio ~9.7). Inert stone chips (0.02–0.05 cm; void ratio ~0.54) were used as packing material for fixed bed to support the biofilm growth. Initially the reactor was operated in suspended growth configuration for a period of 320 days and subsequently shifted to biofilm configuration. Peristaltic pumps integrated with pre-programmed electronic timers were used to regulate the FEED, RECIRCULATION and DECANT operations. The bioreactor was initially operated with designed synthetic wastewater [(mg/l) sucrose, 3000; NH_4Cl, 500; K_2HPO_4, 250; KH_2PO_4, 250; $CaCl_2$, 5; $MgCl_2$, 300; $FeCl_3$, 25; $CoCl_2$, 25; $MnCl_2$, 15; $NiSO_4$, 16; $CuCl_2$, 10; $ZnCl_2$, 10] as feed at an organic loading rate (OLR) of 1.8 kg COD/m^3-day in fed-batch mode [periodic discontinuous batch (PDBR)/sequencing batch (SBR)] with a total cycle period of 24 h comprising 15 min FILL, 23 h REACT, 30 min SETTLE and 15 min DECANT phases after adjusting the pH to 7 so as to support the biofilm formation on the packing medium. At the beginning of each cycle, immediately after withdrawal (earlier sequence), a pre-defined volume (1.5 l) was fed to the reactor during FILL phase and the reactor volume was circulated with outlet-closed loop mechanism at recirculation volume to feed volume ratio of 3 during the REACT phase to achieve a homogeneous distribution of the substrate. Constant COD removal and gas production (~5% variation) were considered as the indicators for stabilized performance of the reactors. Subsequently, reactors were shifted to real field wastewater after adjusting the pH to 6.0 till stable performance was attained in all the experimental variations studied (Table 1).

Prior to feeding, influent pH of wastewater was adjusted to 6.0/7.0, using orthophosphoric acid (88%) or 1 M NaOH. After feeding wastewater, the reactor was sparged with oxygen free nitrogen gas for 2 min to remove possible oxygen accumulated in the headspace of the reactor to ensure complete anaerobic condition. All the experiments were performed at mesophilic (room) temperature (28 ± 2 °C). H_2 gas generated during experiments was estimated using a microprocessor based pre-calibrated electrochemical H_2 sensor (ATMI GmBH Inc., Germany). Volatile fatty acids (VFA) and COD (closed dichromate refluxing method) were determined according to the standard methods [14].

2.3. DNA extraction

The biofilm attached to the stone chips of the biohydrogen reactor was scraped and washed with phosphate buffer (50 mM) twice followed by centrifugation (1200 g for 5 min) to remove suspended particles. Further, bacterial cells were collected by centrifuging the supernant at 8100 g for 10 min and the resulting pellet was suspended in 200 μl TE buffer. Pellet (5 mg) was re-suspended in 0.5 ml of lysis buffer (pH 8, Na_2EDTA, 100 mM; Tris–HCl, 100 mM; sodium phosphate buffer 100 mM) mixed with NaCl (1.5 M) and hexadecyltrimethyl ammonium bromide (CTAB) (1%) followed by lysozyme solution (50 mg/ml) and incubated at 37 °C for 30 min.50 μl of proteinase K solution (20 mg/l) was added and the mixture was re-incubated at 37 °C for 30 min. It was further treated with 200 μl of 20% sodium do-decyl sulphate (SDS). Subsequently, the suspension was agitated at 600 g at 65 °C in water bath for 2 h with end-over-end inversion for every 20 min and later centrifuged at 7800 g for 15 min.