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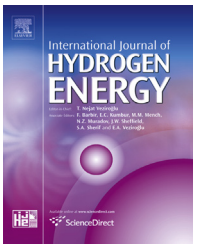

Microbial community analysis of thermophilic mixed culture sludge for biohydrogen production from palm oil mill effluent

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ABSTRACT

The microbial community structure of thermophilic mixed culture sludge used for biohydrogen production from palm oil mill effluent was analyzed by fluorescence *in situ* hybridization (FISH) and 16S rRNA gene clone library techniques. The hydrogen-producing bacteria were isolated and their ability to produce hydrogen was confirmed. The microbial community was dominated by *Thermoanaerobacterium* species (~66%). The remaining microorganisms belonged to *Clostridium* and *Desulfotomaculum* spp. (~28% and ~6%, respectively). Three hydrogen-producing strains, namely HPB-1, HPB-2, and HPB-3, were isolated. 16S rRNA gene sequence analysis of HPB-1 and HPB-2 revealed a high similarity to *Thermoanaerobacterium thermosaccharolyticum* (98.6% and 99.0%, respectively). The *Thermoanaerobacterium* HPB-2 strain was a promising candidate for thermophilic fermentative hydrogen production with a hydrogen yield of 2.53 mol H_2 mol^{-1}hexose from organic waste and wastewater containing a mixture of hexose and pentose sugars. *Thermoanaerobacterium* species play a major role in thermophilic hydrogen production as confirmed both by molecular and cultivation-based analyses.

Introduction

Currently, the disposal of organic wastes and wastewater are an economic burden on communities and industries. Creating a marketable product from these waste products would provide numerous benefits by reducing treatment costs. The production of biohydrogen by dark hydrogen fermentation is a process that can achieve two simultaneous objectives: the production of bioenergy and reduction of pollution [1]. Dark fermentation appears to be an attractive strategy because it can continually produce hydrogen without the need for light,

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and it can use a variety of cheap carbon sources. Several studies have reported successful hydrogen production from wastewater or solid wastes by mixed cultures in batch or bioreactors [2,3]. The advantage of using mixed cultures for biohydrogen production is that no sterilization is required, high adaptive capacity owing to the microbial diversity, the capacity to use a mixture of substrates, and the possibility of obtaining a stable and continuous process. The most common bacteria used in dark fermentation to produce hydrogen are *Clostridium* [4] and *Thermoanaerobacterium* [5]. Normally, hydrogen production yields of 1–2 mol H_2/mol-hexose are obtained with mesophiles, while thermophiles yield more than 2 mol H_2/mol-hexose [6,7]. Hydrogen yields can be improved by increasing acetate end product formation, and decreasing or preventing butyrate formation by using a high temperature fermentation process with thermophiles or extreme thermophiles, operating at temperatures higher than 60 °C [8]. Thermophilic mixed cultures have a high potential as hydrogen producers [7] and they are also able to utilize a wide range of organic wastes [9].

Although hydrogen production from organic wastes or wastewaters by thermophilic mixed cultures can be efficiently produced at the laboratory level, it has been difficult to apply this technology in long term operated bioreactors and for commercial processes. Much work has been done to elucidate the most suitable conditions for hydrogen production, and the major focus has been on optimization of reactor and fermentation conditions [2,10,11]. However, instability during long term operation and relatively high hydrogen production costs are still a problem. Few studies appear to have focused on the microbial community structure in mixed cultures, and how this structure impacts hydrogen production. In addition, the main hydrogen producers are yet to be identified, and it is not known whether the production yield can be increased by the manipulation of the microbial community composition. An important step towards understanding the roles of various bacteria in the hydrogen production process is to identify and quantify the relevant organisms, especially those directly responsible for the hydrogen production activity. The isolation and identification of fermentative hydrogen producers with a high yield and high production rates of hydrogen are also important for developing commercial and sustainable biohydrogen production processes.

This work aims to analyze the microbial community structure of sludge from an anaerobic reactor for continuous hydrogen production from palm oil mill effluent using culture-dependent and independent techniques. Hydrogen-producing bacteria were also isolated and their potential for hydrogen production was investigated.

Materials and methods

Thermophilic mixed culture sludge

Thermophilic mixed culture sludge was taken from a hydrogen-producing anaerobic sequencing batch reactor (ASBR) used for palm oil mill effluent running at a steady condition for 6 months. The ASBR was operated at 60 °C with a hydraulic retention time of 2 days and organic loading rate of 60 gCOD·l^{-1} d^{-1} in continuous mode. The palm oil mill effluent (POME) was fed into the reactor as a substrate. The POME originated from a receiving tank of Trang Palm Oil Co., Ltd. in southern Thailand. The POME had a high temperature (70–90 °C), acidic pH (4.5), was a brownish color, and contained 1.3–2.5% oil, 16–20 g total carbohydrates l^{-1}, a C/N ratio of 95, C/P ratio of 650, 4–5% total solids, 2–4% suspended solids, 70–90 g COD l^{-1}, 830–920 mg total Kjeldahl nitrogen l^{-1}, 97–125 mg total phosphorus l^{-1} and 1.5–2.6 mg iron l^{-1}. The microbiological community of the POME was composed of *Thermoanaerobacterium* sp. and *Microbacterium* sp. The ASBR reactor achieved an optimal volumetric hydrogen production rate of 9.1 l H_2·l^{-1} d^{-1} (17 mmol H_2·l^{-1} h^{-1}) and maximum hydrogen yield of 2.1 mol H_2 mol^{-1}hexose. The hydrogen content and substrate conversion also exceeded 54% and 92%, respectively, with soluble metabolites mainly composed of butyric acid and acetic acid under a steady condition.

DNA extraction

Thermophilic mixed culture sludge from the hydrogen-producing ASBR reactor was taken in sterile 2.0 ml microfuge tubes, immediately closed and stored at −20 °C until further processing. For DNA extraction, the sludge was centrifuged at 10,000 g at 4 °C for 10 min, and the resulting pellet was resuspended in 100 μl of 10 mmol l^{-1} Tris–HCl, pH 8.5. Total genomic DNA was subsequently extracted by using the Ultraclean Soil DNA Kit (MoBio Laboratory Inc., USA), which has been described previously for the extraction of DNA from anaerobic sludge [12]. DNA was stored at −20 °C until further processing.

Clone library construction and phylogenetic analysis

The total DNA extraction was used to amplify the almost full length 16S rRNA gene with 27f and 1527r primers for *Bacteria* [13] and Arch21f and Arch958r for *Archaea* [14]. The amplicons were purified using the E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and ligated into a Topo TA cloning vector (Invitrogen, CA) using the manufacturer's protocol. The recombinant plasmids were transformed to competent *Escherichia coli* DH5α cells. A total of 113 colonies were selected for inoculation in a 5.0 ml LB medium containing 50 mg Ampicillin l^{-1}. After 24 h of incubation at 37 °C, the plasmids were recovered and subjected to a PCR analysis using the primer set of M13f (5′-GTAAAACGACGGCCAG-3′) and M13r (5′-CAGGAAACAGCTATGAC-3′) to check the presence and size of the inserts. The full length of 16S rRNA gene inserts were reamplified and analyzed by DGGE using the protocol described according to Prasertsan et al. [3] in order to identify the various clone types. Nucleotide sequences were compared with those in GenBank databases with the BLAST program [15]. Sequences were checked for possible chimera using the chimera check program of the ribosomal database project (http://rdp8.cme.msu.edu/cgis/chimera). Data processing for obtained sequences by alignment, phylogenetic trees construction and bootstrapping analysis were performed according to Prasertsan et al. [3].

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