Bioresource Technology 102 (2011) 8445–8456

Contents lists available at ScienceDirect

Bioresource Technology

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

Application of molecular techniques on heterotrophic hydrogen production research

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ARTICLE INFO

Article history:
Received 29 November 2010
Received in revised form 16 February 2011
Accepted 17 February 2011
Available online 21 February 2011

Keywords:
Hydrogen production
Molecular techniques
16S rDNA
Fe-hydrogenase gene
PCR

ABSTRACT

This paper reviews the application of molecular techniques in heterotrophic hydrogen production studies. Commonly used molecular techniques are introduced briefly first, including cloning-sequencing after polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP), fluorescence *in situ* hybridization (FISH) and quantitative real-time PCR. Application of the molecular techniques in heterotrophic hydrogen production studies are discussed in details, focusing on identification of new isolates for hydrogen production, characterization of microbial compositions in bioreactors, monitoring microbial diversity variation, visualization of microbial distribution in hydrogen-producing granular sludge, and quantification of various microbial populations. Some significant findings in recent hydrogen production studies with the application of molecular techniques are discussed, followed by a research outlook of the heterotrophic biohydrogen field.

1. Introduction

Energy and environment are essential for sustainable development of the global prosperity. Currently, over 80% of energy supply is dependent on fossil fuels, which cause the deterioration of environment and rapid exhaustion of natural energy sources (Guo et al., 2010). This has led to the search for alternative energy sources, among which hydrogen has attracted much attention recently. As a clean energy, producing only water after combustion, hydrogen may become an alternative to fossil fuels in the future. It also has a high energy yield of 122 kJ/g, which is about 2.75 times that of fossil fuels (Kim et al., 2006a).

At present, hydrogen is commercially produced by either thermocatalytic reformation of hydrocarbons or electrolysis of water, both of which are highly energy consuming and unsustainable processes (Das and Veziroglu, 2008). Heterotrophic biological production of hydrogen has, however, attracted research interests due to its potential ability of degrading organic pollutants which serve as carbon and energy sources for the microbes during harvesting hydrogen (Li and Fang, 2009). Heterotrophic hydrogen production is often classified into two categories depending on whether light is required, i.e. dark fermentation and photo fermentation (Levin et al., 2004). Dark fermentation converts organic pollutants into hydrogen by dark fermentative bacteria in the absence of light, producing organic acids, mainly acetate and butyrate, and alcohols as by-products. Photo fermentation is potentially able to convert acids and alcohols, which are the by-products of dark fermentation, into hydrogen by photosynthetic bacteria using light as energy source. Dark fermentation has a high production rate of hydrogen, but with low hydrogen yield, converting no more than 40% of the chemical energy in the organic pollutants into hydrogen (Li and Fang, 2007). In comparison, photo fermentation produces little organic residues, resulting in higher hydrogen yield, but has much lower hydrogen production rate than dark fermentation (Lee et al., 2010).

Various factors have been studied for heterotrophic hydrogen production including source of inoculation, feeding substrates, reactor design and operating conditions such as pH, temperature and hydraulic retention time (HRT) etc. With the development of molecular techniques, identification and quantification of microorganism communities involved in hydrogen production become more convenient, effective and accurate. The nucleic acid based techniques have been widely used in heterotrophic hydrogen production studies in the past decade, which contributed much to identification of the new isolated hydrogen-producing bacteria, exploration of the metabolic functions and interactions of different species in hydrogen production system, and investigation on the effects of operational factors on microbial communities. The application of molecular techniques will thus help to optimize the operational conditions of the bioreactors, improve the reactor stability, and increase the hydrogen production rate and yield.

This article aims to review the application of molecular techniques in heterotrophic hydrogen production studies. Commonly

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doi:10.1016/j.biortech.2011.02.072

used molecular techniques are introduced briefly first, including cloning-sequencing after polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993), terminal-restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997), fluorescence *in situ* hybridization (FISH) (Wagner et al., 2003) and quantitative real-time PCR (qRT-PCR) (Zhang and Fang, 2006). Applications of the above molecular techniques in analysis of microbial communities in hydrogen production bioreactors are discussed in details, focusing on identification of new isolates for hydrogen production, characterization of microbial compositions in bioreactors, monitoring microbial diversity variation, visualization of microbial distribution in hydrogen-producing granular sludge, and quantification of various microbial populations. Four tables were compiled for heterotrophic hydrogen production data scattered in literature in terms of aforementioned respective applications except visualization of microbial distribution in granular sludge, which had very limited reports. Data summarized in tables include fermentation type (dark or photo-fermentation), seed sludge (or isolation source for Table 1), reactor design (batch or type of continuous reactor), culture volume (l), substrate, maximum volumetric hydrogen production rates (l-H_2/l/h), hydrogen yield (mol H_2/mol carbohydrates consumed for dark fermentation and compared to stoichiometry (%)), cell density (g-VSS/l), molecular techniques used and microbial analysis results.

2. Molecular techniques

The starting point for the molecular methods and related procedures is the extraction of nucleic acids. The reliability of the molecular techniques depends on quality and representativeness of RNA/DNA extracted from sludge samples in the reactors. The DNA extraction process is composed of cell lysis, contamination removal, solvent extraction, precipitation and purification (Miller et al., 1999). The amount of nucleic acid (as expressed by A_{260}) and purity (as expressed by the ratios of A_{260}/A_{280} for protein contamination and A_{260}/A_{230} for salt contamination) are measured spectrophotometrically using the conventional UV–visible spectrometer or the more advanced spectrometers, such as Nanodrop. The integrity of rRNA (containing 5S, 16S and 23S) and genomic DNA could be visually checked by conducting electrophoresis using agarose gel. The most commonly used phylogenetic biomarker is 16S rRNA gene (16S rDNA) which has a huge database available (over 338, 193 of 16S rDNA sequences in GenBank as of Nov. 27, 2010).

The extracted DNA is subjected to PCR amplification using "universal" primers or primers designed to amplify rRNA genes from particular group of organisms. The PCR cycle takes place in three steps: denaturing, annealing and extension. The crucial part of a PCR is the selected primer set targeting genes of interest which cover special taxonomic or functional groups. These primers may be individually designed based on the alignments of relevant DNA sequences, or simply adapted from the literature. The bacteria-specific primers, such as the sets of EUB8F and UNIV1492R (Zhu et al., 2008), EUB968F and UNIV1392R (Hung et al., 2008), EUB341F and UNIV518R (Akutsu et al., 2008), and EUB357F and UNIV518R (Shin et al., 2004), were commonly used in the microbial analysis in hydrogen production studies.

The selected primer sets may target the sequences at various taxonomic levels, from domain to division, subdivision, class, family, genus, species, and even strain. The PCR products contain a mixture of multiple copies of the same segment amplified at the selected taxonomic level. The PCR products can be cloned and then sequenced to identify species. They can also be analyzed by various techniques, such as DGGE or T-RFLP, which may separate PCR products originating from different DNA sequences of various species in the sample.

2.1. Cloning and sequencing

Cloning of PCR products is to separate the PCR fragments of the same length but of different sequences. Cloning is composed of three steps: ligation, transformation and host cell reproduction. Several commercial kits are available for DNA cloning, such as the pGEM-T cloning kit and the TA cloning kit.

Taking the TA cloning kit as an example, PCR products are firstly inserted into the plasmids under the action of the ligase. After ligation, the plasmids with PCR product inserts are transformed into *Escherichia coli* competent cells. Each cell carrying the plasmid with the PCR product insert forms a single white colony (called the clone) on the solid discriminative medium. Whereas, each cell carrying the plasmid without the PCR product insert forms a blue one, and each cell carrying no plasmid does not form any colony. A DNA library consists of the selected white colonies. The insert in plasmids of the white colonies may be recovered using PCR with a primer set targeting the sequence on the plasmids which locate at the two sides of the insert or the original primer set which is used to generate PCR products initially. The whole plasmid could be extracted after further cultivation of the colonies in liquid medium. The PCR product obtained using the first method, or the plasmids obtained using the second methods, will be sequenced for further identification of the sequences.

Sequencing of the full 16S rDNA (about 1540 bp) is preferred for microbial identification, especially for oligonucleotide probe design and classification of the pure culture. Once a sequence database of a clone library is established, the microbial diversity can be determined with reference to the published sequences of the pure cultures and environmental samples. The similarity analysis of DNA sequences is greatly facilitated by a number of rDNA sequence databases, such as GenBank (http://www.ncbi.nlm.nih.gov/blast/) and the ribosomal database project (RDP, http://rdp.cme.msu.edu/), and powerful software packages, including MEGA 2.1 (Kumar et al., 1993) and ARB (Strunk and Ludwig, 1997). By using the Blast program in GenBank, species closely related to the obtained sequences are listed in the similarity order (Altschul et al., 1990). A primary taxonomy position of the species represented by obtained sequences may also be given. More accurate taxonomy analysis of DNA sequences could be conducted by construction of phylogenetic trees.

The obtained sequence information in a clone library can be used to evaluate species diversity or richness of microbial communities in bioreactors, and to design specific probes/primers for characterization/quantification of a certain microorganism. But the cloning method is time-demanding and less applicable for analysis of a larger set of samples, such as monitoring the changes of a microbial community over time (Sanz and Köchling, 2007).

2.2. Denaturing gradient gel electrophoresis (DGGE)

PCR amplification produces DNA segments with the same size but different sequences. These segments may be separated in an acrylamide gel, but not in an agarose gel, having a linear ascending gradient of denaturants, usually urea and formamide (Muyzer et al., 1993). This so-called DGGE method is based on the differences of the electrophoresis mobility of the partially denatured double-stranded DNA fragments in the polyacrylamide gel.

The universal primer for Bacteria domain 341F-518R (Fang et al., 2006a) is usually used in hydrogen production studies. After staining, DNA fragments will appear as separated bands on the gel. Each DGGE band is derived from one specific species in the original samples. Thus the band number of a DGGE profile provides a quick