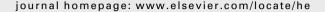
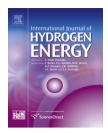


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Fermentative hydrogen production in recombinant Escherichia coli harboring a [FeFe]-hydrogenase gene isolated from Clostridium butyricum

Sanjukta Subudhi*, Banwari Lal*

Environmental and Industrial Biotechnology division, The Energy and Resources Institute (TERI), Habitat Place, Darbari Seth Block, Lodhi Road, New Delhi 110003, India

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ABSTRACT

The [FeFe]-hydrogenase (hydA) from Clostridium butyricum TERI BH05-2 strain was isolated to elucidate its molecular characterization. A 1953 bp DNA fragment encompassing the ORF and the putative promoter region of hydA gene was PCR amplified and subcloned into pGEM®-T-Easy cloning vector (pGEM®-T-hydA). The hydA DNA sequence revealed the presence of a 1725 bp length ORF (including the stop codon) encoding 574 amino acids with a predicted isoelectric point and molecular mass of 6.8 and 63097.67 Da, respectively. The hydA ORF was PCR amplified from pGEM®-T-hydA and inserted into a prokaryotic expression vector to create a recombinant plasmid (pGEX-5X-hydA) and transformed into Escherichia coli BL-21. The recombinant E. coli BL-21 was investigated for fermentative hydrogen production under anaerobic condition from glucose. Heterologous expression of the Clostridium butyricum hydA resulted in 1.9 fold increase in hydrogen productivity as compared to that from the wild type strain, C. butyricum TERI BH05-2. The hydrogen yield of the recombinant strain was 3.2 mol H₂/mol glucose, 1.68 fold higher than the wild type parent strain.

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1. Introduction

Hydrogen (H₂) is renewable, efficient, and environmentally benign and thus considered as a promising energy source for the future [1]. Anaerobic microorganisms have evolved unique mechanisms for hydrogen production. The facultative and strict anaerobic bacteria such as Escherichia coli, Enterobacter species [2,3] and Clostridia species [4,5], respectively, have been reported for fermentative conversion of organic substrates to hydrogen.

In Clostridia species fermentative hydrogen production proceeds from the anaerobic metabolism of pyruvate by the

following electron transfer chains: pyruvate: ferredoxin oxidoreductase (PFOR), NADH: ferredoxin oxidoreductase (NFOR), and hydrogenase [6]. Pyruvate: ferredoxin oxidoreductase (PFOR) from several anaerobic or microaerobic species of microorganisms reduces ferredoxin as it breaks down pyruvate to acetyl-CoA. Recently, Agapakis et al. have reported for creation and characterization of a synthetic electron transfer circuit that couples hydrogen evolution with the breakdown of glucose in *E. coli* via heterologous expression of PFOR, Ferredoxin, and [FeFe]-hydrogenase [7].

Hydrogenase enzyme is principally the prime candidate in these fermentative microbes which catalyzes the reduction of

^{*} Corresponding authors. Tel.: +91 11 24682100; fax: +91 11 24682144. E-mail addresses: ssubudhi@teri.res.in (S. Subudhi), banwaril@teri.res.in (B. Lal).

protons into hydrogen. Based on the sequence of constituent polypeptides and the structure of the metal center active site, hydrogenases are categorized into three groups: [NiFe]hydrogenase, [FeFe]-hydrogenase and [Fe]-hydrogenase [8,9]. In E. coli the native [NiFe]-hydrogenases are coupled to NADH with a reducing potential of -320 mV, while [FeFe]hydrogenases are partnered with the electron carrying protein ferredoxin which can have a significantly stronger reducing potential, close to that of the H_2/H^+ (-420 mV) [10]. Hence, [FeFe]-hydrogenases thermodynamically favor hydrogen production. Compared to [NiFe]-hydrogenase, [FeFe]-hydrogenase is 10 times more active as a hydrogen producer [11]. Clostridial hydrogenases belong to the group of [FeFe]-hydrogenase most of which are cytoplasmic, soluble and monomeric [12]. [FeFe]-hydrogenases from Clostridium species contain several 'ferredoxin-like' domains which enhances hydrogenase interaction with other ferredoxins and provides an electron transport channel toward the hydrogenase active site [13]. Among few hydrogenases those are characterized the [FeFe]-hydrogenase I of Clostridium pasteurianum is the most extensively studied enzyme [12,13]. Furthermore, [FeFe]-hydrogenases have been characterized and recombinant strains have been constructed for several species of Clostridium; C. acetobutylicum, C. pasteurianum, C. tyrobutyricum, C. perfringens and C. paraputrificum [14–19].

[FeFe]-hydrogenases serve as excellent enzymatic modules for recombinant expression in a synthetic system because of their high hydrogen production activity, conserved structure including simple maturation pathway [7]. Heterologously expressed [FeFe]-hydrogenases have been characterized from several species in vitro and in vivo [20—22]. In vivo heterologous expression of [FeFe]-hydrogenase alone is sufficient for small, measurable hydrogen production from E. coli and this hydrogen production can be enhanced with the co-expression of ferredoxins from several organisms [21]. Mishra et al. isolated a novel [Fe]-hydrogenase gene from Enterobacter cloacae IIT-BT 08 [23]. Overexpression of this [Fe]-hydrogenase in E. coli BL-21 resulted in 3.12 mol of hydrogen yield from 1 mol of glucose [24].

In the present study, we report the isolation, molecular characterization and heterologous expression of [FeFe]-hydrogenase gene from *C. butyricum* TERI BH05-2, in *E. coli* BL-21.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Clostridium butyricum TERI BH05-2, a dark fermentative hydrogen producing bacterium, was isolated from sediment samples collected from Yamuna river (contaminated with organic pollutants), flowing below the Delhi—Noida-Direct flyway, New Delhi, India (28_3704000N 77_1502100E) [25]. pGEM®- T-Easy (Promega, USA) plasmid was used for cloning and sequencing of C. butyricum TERI BH05-2 hydA gene. Plasmid pGEX-5X-3 (Pharmacia, USA) was used to over express the hydA gene. Selection for the presence of plasmids was carried out in the presence of 100 µg/ml ampicillin. For DNA extraction, batch cultures of C. butyricum TERI BH05-2

strain were grown anaerobically at 37 °C in modified DMI medium. The modified DMI medium was composed of (per liter): 5.24 g of NH₄NO₃, 6.72 g of NaHCO₃, 0.087 g of K₂HPO₄, 0.5 g of MgCl₂, 0.0075 g of MnSO₄, 0.025 g of Na₂S, 0.02 g of FeSO₄, 4 g of malt extract, 4 g of yeast extract and 10 g of glucose [25]. Modified DMI medium is a modified form of DMI medium [26]. The DMI medium was composed of (g/l): NH₄HCO₃, 5.24; NaHCO₃, 6.72; K₂HPO₄, 0.125; MgCl₂, 0.1; MnSO₄, 0.015; Na₂S, 0.5; FeSO₄, 0.01; resazurin, 0.01; and carbon source as a substrate, 17.8. E. coli BL-21 (Promega, USA) cells were grown anaerobically at 37 °C in Luria Bertani medium (5 g yeast extract, 10 g peptone and 10 g NaCl per liter). Recombinant E. coli BL-21 was cultivated in Luria Bertani medium supplemented with ampicillin (100 μg/ml).

2.2. DNA isolation and manipulation

RNA free genomic DNA was extracted from C. butyricum TERI BH05-2 by employing the method as described previously [27]. The plasmid DNA was prepared from E. coli cells with an alkaline lysis technique using GETTM plasmid miniprep kit (G-Biosciences, USA). The PCR amplified hydA ORF was gel purified and digested with BamHI restriction enzyme by incubating at 37 °C for 2 h. The digested hydA gene fragment was ligated into BamHI digested pGEX-5X-3 prokaryotic expression vector.

2.3. Nucleotide sequence determination of C. butyricum TERI BH05-2 strain hydA gene

A pair of oligonucleotide PCR primers: UpSt-FW/RV-1 (Table 1) were designed, based on the published nucleotide sequences of [FeFe]-hydrogenase genes (hydA coding sequence with its upstream putative promoter sequence) from other C. butyricum bacterial strains, as available in the NCBI database. A 1953 bp DNA fragment was amplified from genomic DNA of C. butyricum TERI BH05-2 strain by PCR using the above mentioned designed PCR primer pair. The PCR conditions consisted of an initial denaturation step for 2 min at 95 °C, 30 cycles of repeated denaturation step at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension of 10 min at 72 °C. The PCR amplified DNA fragment was gel purified and cloned into pGEM®-T-Easy plasmid vector. The ligated plasmid construct was transformed into the E. coli JM109 strain as per the manufacturer's instructions (Promega, USA). Presence of insert was confirmed by restriction enzyme digestion and nucleotide sequencing. The recombinant plasmid construct was designated as pGEM®-T-hydA (Fig. 1). ORF finder tool (http://www.ncbi.nlm.nih.gov) was employed for determination of hydA ORF sequence. The instructions were followed as described in the above mentioned web site. BLASTN was used to search for nucleotide identity of hydA coding region. BLASTP was used to search for deduced amino acid identity of hydA coding region. The theoretical isoelectric point (pI) and molecular weight (MW) of hydA gene encoding hydrogenase enzyme were determined by employing web-based software (http://br.expasy.org/cgi-bin/pi_tool). ScanProsite tool (http:// expasy.org/tools/scnpsit3.html) was employed for functional domain analysis of hydA amino acid sequence.

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